THE MOLECULAR CHARACTERIZATION OF SWINEPOX VIRUS

By

ROBERT F. MASSUNG

A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

1991

ACKNOWLEDGEMENTS

I would like to thank Dick Moyer for his unending support, advice and encouragement. He was always there to give me direction when I needed it, while also providing the freedom necessary for me to develop as a scientist. I also want to thank Sue Moyer for the many contributions she has made towards my work, and also the personal support she has provided to myself and my family throughout these difficult years of graduate school.

I thank all the members of the Moyer lab group for their input and for making life in the lab enjoyable. I particularly want to thank Joyce, the other "pig person" in the lab, for her help on numerous facets of my work. I appreciate the sequencing help from Duke and Dot, without which I would still be running gels today.

I would like to thank the members of my committee, Paul Gulig, Henry Baker and Carl Feldherr, for their advice and support, and particularly Dr. Felherr for all his help with my previous oocyte work. I also appreciate all the assistance I received from other faculty members and graduate students, too numerous to mention. In particular, I want to thank Rich Condit for his assistance with the sequencing project and for making me VAX literate.

I thank my family, for without them nothing I have done would have been possible. I thank my parents, Dorothea and Fred, for never giving up on me and always being there when I needed them. Lastly, and most importantly, I thank my wife, Marcia, for her love, sacrifices and constant support through the good and bad times, and my children, Robbie, Breanne and Valerie, for helping me maintain the proper perspective and allowing me to realize what is really important in life.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

KEY TO ABBREVIATIONS

A	Adenosine
A_{260}	$Absorbance_{260}$
AA	Amino acid
bp	Basepair
β-ME	Beta-mercaptoethanol
BSA	Bovine serum albumin
C	Cytosine
CAR	Cytosine arabinoside
Ci	Curie
CPE	Cytopathic effect
dATP	Deoxyadenosine 5'-triphosphate
dCTP	Deoxycytidine 5'-triphosphate
dGTP	Deoxyguanosine 5'-triphosphate
DNA	Deoxyribonucleic acid
dT	Deoxythymidine
DTT	Dithiothreitol
dTTP	Deoxythymidine 5'-triphosphate
EDTA	Disodium ethylenediamine tetraacetate
FBS	Fetal bovine serum
G	Guanosine
g	Gravity
HCl	Hydrochloric acid

HEPES	N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid
hr	Hour
ITR	Inverted terminal repeat
kb	Kilobase
kbp	Kilobasepair
kD	Kilodalton
μCi	Microcurie
μg	Microgram
μl	Microliter
mg	Milligram
ml	Millileter
mM	Millimolar
mmol	Millimole
min	Minute
mRNA	Messenger ribonucleic acid
nm	Nanometer
NP40	Nonidet P-40
O.D.U.	Optical density unit
PEG	Polyethylene glycol
PBS	Phosphate buffered saline
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulfate
TBE	Tris(99mM)/borate(99mM)/EDTA(1mM)
TE	Tris(50 mM)/EDTA(1mM), pH8.0
Tris	Tris(hydroxymethyl)aminomethane

V	Volt
V-hr	Volt-hour

Abstract of Dissertation Presented to the Graduate School of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

THE MOLECULAR CHARACTERIZATION OF SWINEPOX VIRUS

By

Robert F. Massung

May, 1991

Chairman: Richard W. Moyer
Major Department: Immunology and Medical Microbiology

Swinepox virus, the prototype and only member of the Suispoxvirus genus, has not been previously characterized at the molecular level, and its classification is based solely on in vitro neutralization assays. We have analyzed the DNA of this virus and demonstrate that the genome is 175 kilobases in size and, like the more commonly studied Orthopoxvirus, Avipoxvirus and Leporipoxvirus genera, is terminally cross-linked and contains inverted terminal repetitions. The length of the inverted repeats are shown to be approximately 5 kilobases, and the DNA comprising these repeats are unstable, probably due to the presence of a variable number of direct repeats 70 basepairs in length. Restriction endonuclease cleavage maps for the enzymes HindIII, AvaI, HaeII, KpnI, BglI, SalI and XhoI are also presented.

The growth characteristics of swinepox virus in tissue culture were examined by light microscopy and revealed both a delayed and different cytopathology compared to that of vaccinia virus, the prototype poxvirus. Gene expression was characterized at the levels of DNA accumulation, RNA transcription and protein synthesis, and the kinetics of each facet of expression are shown to be considerably delayed when compared to vaccinia.

Studies based on low stringency hybridizations of radiolabeled swinepox virus DNA to Southern blots containing DNA of representative members of various other poxvirus genera revealed no DNA homology at this level of resolution. The antigenic relateness between swinepox and vaccinia virus was analyzed by immunoprecipitations and revealed little, if any, cross-reactivity.

We have analyzed the genetic content of swinepox virus by sequencing the DNA from selected regions of the genome. A region sequenced from within the central core of DNA was shown to have a high degree of homology to the analogous region of vaccinia virus DNA. However, the DNA sequence from the terminal region of the swinepox virus genome was remarkably dissimilar to the same region of vaccina DNA. The combined data indicates that swinepox virus is unique from other poxviruses characterized to date and confirms the previous classification of swinepox virus into a separate genus within the poxvirus family.

CHAPTER 1

INTRODUCTION

The poxviruses comprise a large and diverse family of viruses which, as a group, are capable of infecting a wide variety of hosts ranging from insects to man. The severity of natural infections vary widely from highly virulent to subclinical. Some, such as Variola in man or Ectromelia in mice, frequently result in host death. Others, such as swinepox virus (SPV), result in a mild, often inapparent infection. Several other poxviruses, including Shope fibroma virus (Shope, 1932), Yaba tumor virus (Bearcroft and Jamieson, 1958) and Molluscum contagiosum (Brown et al. 1981), have been implicated in tumor induction. Likewise, the range of host specificity varies drastically among the individual members of the family. Many, such as SPV and ectromelia, are limited to a single host species, whereas others, such as vaccinia virus, the most well studied member of the family, demonstrate a very wide host range. The reasons responsible for the variations noted among the various members of the poxvirus family with regard to host range and pathogenicity are not well understood at the molecular level, partly because poxviruses, and the expression of their genes, are quite complex.

Poxviruses are among the largest animal viruses. The virions are approximately 250 x 350 nm in size and are often referred to as brick shaped. More than 100 polypeptides can be resolved by two dimensional electrophoresis of purified virions (Essani and Dales, 1979; Oie and Ichihashi, 1981). The double stranded DNA of these viruses is packaged within a biconcave core composed of a lipoprotein bilayer. In the vertebrate poxviruses, two stuctures of unknown function termed lateral bodies are located outside the core and within the concavities. The core and lateral bodies are surrounded by an outer membrane or envelope. Upon infection of a susceptible host, the first event to occur is the removal of this outer membrane via fusion with either the host cell membrane or an endocytic vesicle membrane (Chang and Metz, 1976; Dales, 1973; Dales and Kajioka, 1964). The removal of this outer membrane appears to be the signal required for the initiation of viral gene expression because expression can also be induced in vitro by removing the outer membrane by treatment with a nonionic detergent and a reducing agent (Paoletti, 1977).

Poxvirus genomes range in size from approximately 130 to 300 kb, and every one examined to date has termini that are covalently cross-linked. The termini of these viruses also contain inverted terminal repetitions (ITRs) of variable length, the only possible notable exception being variola, the causative agent of smallpox (Dumbell and

Archard, 1980; Esposito and Knight, 1985). In contrast to most members of the poxvirus family, vaccinia has been intensively studied at the molecular level, and the entire genome of the Copenhagen strain and most of the Western Reserve strain have recently been sequenced. The Copenhagen strain has a genome of 191.6 kilobasepairs, ITRs of 12 kbp, a nucleotide composition of 66.6% A+T, and potentially encodes for greater than 200 polypeptides (Goebel et al., 1990). Genes are transcribed from both strands. There are no introns, and many of the open reading frames are arranged in a head to tail tandem orientation with very short untranscribed intergenic regions (Earl and Moss, 1989). Most of the genes essential for replication are clustered in the central area of the genome, while the regions near the termini are nonessential, at least in tissue culture, and much more variable in terms of genetic content (Mackett and Archard, 1979; Esposito and Knight, 1985).

Poxvirus gene expression is classically and for simplicity, divided into two phases: early and late. Early gene expression initiates rapidly after uncoating, utilizes enzymes packaged within the virion, and must occur prior to subsequent late gene expression. Early messages are capped, polyadenylated, and have discrete 5' and 3' termini (Moss, 1990). The beginning of late gene expression coincides with the initiation of DNA replication and results in the synthesis of gene products required for viral morphogenesis

(Dales and Pogo, 1981; Moss, 1990). Late messages are also capped and polyadenylated, but also include a unique nonencoded 5' poly(A) leader and heterogeneous 3' ends due to a deficiency in transcriptional termination (Bertholet et al., 1987; Schwer et al., 1987). However, recently a subset of genes refered to as "intermediate" have been identified that are expressed after the initiation of replication but appear to be independent of replication per se, requiring only a naked template (Vos and Stunnenberg, 1988; Keck et al., 1990). Therefore, it seems likely that a much more complex cascade model of gene expression, as noted for other viral families such as the herpesviruses (Roizman and Batterson, 1985), is emerging for the poxviruses.

Poxvirus gene expression and the kinetics of DNA replication in cell culture has been best characterized for the Orthopoxvirus genus, with most of the studies performed with vaccinia virus. For vaccinia, early gene expression begins rapidly after uncoating, replication begins at 2-4 hr postinfection, and late gene expression is observed as early as 3-5 hr postinfection (Carrasco and Bravo, 1986; Kates and McAuslan, 1967a; Moss and Salzman, 1968; Munyon and Kit, 1966; Pennington, 1974). Some poxviruses of other genera, such as the leporipoxviruses, have been shown to exhibit kinetics similar to vaccinia (Pogo et al., 1982). Others, such as the avipoxviruses and tanapoxviruses, have been reported to be "slow" relative to vaccinia but have not been

well characterized at the molecular level (Knight et al.1989; Schnitzlein et al.1988).

One of the unique characteristics common to all members of the poxvirus family is that the entire lifecycle of the virus occurs in the cytoplasm of the host cell. A consequence of a cytoplasmic site of development is that these complex viruses encode many enzymes, including those needed to initiate viral transcription such as a DNA dependent RNA polymerase (Kates and McAuslan, 1967; Munyon et al., 1967), capping and methylating enzymes (Ensinger et al., 1975; Martin and Moss, 1975; Martin et al., 1975; Martin and Moss, 1976; Moss et al., 1975b; Moss et al., 1976; Shuman et al., 1980; Wei and Moss, 1974; Wei and Moss, 1975), and a poly (A) polymerase (Moss et al., 1973; Moss and Rosenblum, 1974; Moss et al., 1975a). Poxviruses also encode numerous enzymes involved in DNA metabolism including a DNA polymerase (Challberg and Englund, 1979; Earl et al., 1986; Jones and Moss, 1985; Traktman et al., 1984), topoisomerase (Bauer et al., 1977; Shuman and Moss, 1987), and ribonucleotide reductase (Slabaugh et al., 1988; Tengelsen et al., 1988), and they express these genes at early times. The majority of late gene expression is dedicated to the synthesis of viral structural proteins, viral enzymes that are packaged within the virions, and other factors involved in the morphogenesis of immature

virions to mature viral particles (Bauer et al., 1977; Moss et al., 1973).

The potential for vaccinia to be used as a multipurpose live vaccine vector has been the subject of much interest within the last decade (Tartaglia et al., 1990; Moss and Flexner, 1987). More recently, fowlpox virus, a member of the Avipoxvirus genus, has been suggested as a suitable vector strain (Boyle and Coupar, 1988; Taylor and Paoletti, 1988). In theory, any poxvirus has the potential to be adapted and manipulated to serve as a live virus vaccine vector. However, there are certain features inherent in many poxviruses that are disadvantageous with respect to their use as vaccine vectors. One such problem with some members is their general promiscuity, or lack of strict species specificity, which in turn might lead to the spread of the vector in the field to animal species other than those for which the vaccine is intended. A more important concern is the innate virulence of many of these viruses, such as vaccinia, which normally produces an acute systemic infection, and occasionally results in post-vaccinial encephalitis (PVE) with a mortality rate of 25% (Fenner et al., 1989). The ideal poxvirus to be selected for vaccine development would elicit a strong immune response with limited virulence, and be capable of infecting only the species to be vaccinated, which would eliminate the possibility of spread of the virus through the environment

to non-intended hosts. One poxvirus which potentially fulfills all these criteria is swinepox virus (SPV), the type species and only reported member of the genus Suipoxvirus (Matthews, 1982). This virus has previously been uncharacterized except with regard to the ultrastructure of the virion, and the pathology and histopathology of infections in swine (Cheville, 1966; Conroy and Meyer, 1971; Meyer and Conroy, 1972; Kasza et al., 1960). The classification of SPV was based primarily on serological data, specifically the reported lack of neutralizing antibodies that are cross-reactive with members of the other poxvirus genera (Shope, 1940; Schwarte and Biester, 1941). Swinepox virus is species specific in that it only infects swine (Datt, 1964), and the clinical presentation is that of a mild, self-limiting infection, with lesions detected only in the skin and regional lymph nodes (Kasza and Griesemer, 1962). This is in contrast to numerous other poxviruses such as vaccinia, capripox, ectromelia, and fowlpox viruses, for which infection of susceptible hosts results in spread to and lesions of internal organs. However, before swinepox virus can be explored as a potential live virus vaccine, a physical, molecular and biological characterization of the virus and its development within the host is required. This information would also be very useful in terms of taxonomically classifying this virus, allowing us to determine the evolutionary divergence of swinepox virus as

compared to the other members of the poxvirus family, and extend our overall knowledge of the biological properties of the entire poxvirus family.

The work presented here provides the first extensive examination of swinepox virus infections at the biological and molecular levels. The restriction endonuclease cleavage maps for seven enzymes within the SPV genome have been determined. The complexity of the viral genome has also been determined, and evidence is presented that the termini of the DNA are covalently closed. These data are consistent with the presence of both tandem and inverted terminal repeats near the termini of the genome. These studies have demonstrated that many of the distinct genomic features common to the members of the poxvirus family have been conserved within another, and until now, unexamined member of the Chordopoxviridae subfamily of the family Poxviridae. The work presented suggests that there is no gross detectable homology at the DNA level between SPV and the ortho-, lepori-, avi- or entomopox virus groups via hybridization data. The data also demonstrate that there is little or no antigenic cross-reactivity between vaccinia and swinepox virus as evidenced by immunoprecipitations with appropriate antisera. This work shows that vaccinia and SPV infections of a given tissue culture cell line differ dramatically, both in the eventual outcome of the infection (plaques for vaccinia versus foci for SPV) and in the time

required for cytopathic effects (CPE) to become visible. Also examined are the kinetics of DNA accumulation, protein synthesis, and RNA transcription. These studies demonstrate that the development of swinepox virus within its host cell is delayed when compared to that of the ortho- and leporipoxviruses. The DNA sequence of approximately 2.8 kilobases from the central conserved core region and 7.6 kilobases near the left terminus of the SPV genome is presented. The open reading frames deduced from the core region sequence are highly conserved with regard to other paxviruses, while the terminal sequence demonstrates little conservation. Therefore, despite the similarity in terms of genomic features and the conservation noted in the sequence of the conserved region, the evidence presented here indicates that SPV is indeed unique from vaccinia and other poxvirus family members, and confirms the previous classification of SPV into a separate genus.

CHAPTER 2

MATERIALS AND METHODS

Viruses and Cell Culture

Vaccinia virus (IHD-J strain) and swinepox virus (Kasza strain) were obtained from the American Type Culture Collection. Vaccinia strain IHD-W was obtained from B. Pogo (Mt. Sinai School of Medicine), Shope fibroma virus from G. McFadden (University of Alberta), myxoma virus from Dr. D. Strayer (Univ. of Texas Health Science Center), Amsacta moorei entomopox virus (AmEPV) from R. Hall and F. Hink (Ohio State University), and fowlpox virus (CEVA strain) from D. Tripathy (University of Illinois-Urbana). Vaccinia was grown in either Rat 2 or porcine kidney (PK-15) cells, swinepox virus in PK-15 cells, and myxoma and Shope fibroma virus in rabbit kidney (RK-77) cells. Each of these cell lines was maintained as monolayers in Eagle's MEM (F-11; GIBCO laboratories) supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 units penicillin, 100 μg streptomycin and 0.1 mg pyruvate per ml. Fowlpox virus (FPV) was grown in quail (QT-35) cells maintained as monolayers in Opti-MEM media (GIBCO Laboratories) supplemented with 5% fetal bovine serum, 2 mM glutamine, 100 units penicillin, 100 μg streptomycin and 0.1 mg pyruvate per ml. AmEPV was

grown in gypsy moth (IPLB-LD-652) cells obtained from Ed Dougherty (Insect Pathology Laboratory, USDA, Beltsville, MD) which were cultured in Excell 400 (J R Scientific) supplemented with 5% fetal bovine serum.

Viral DNA Isolation

Viral DNA was isolated by one of two protocols. For vaccinia, Shope fibroma and myxoma viruses, virions were initially purified by the method of Moyer and Rothe (1980). The procedure involved first pelleting the virus through a pad of 1.46 M sucrose followed by banding of the virus on potassium tartrate gradients. The viral band was then diluted with PBS and pelleted through another 1.46 M sucrose pad in order to remove the potassium tartrate. The DNA was subsequently extracted by lysis of the purified virions in a solution containing 1% sodium dodecyl sulfate (SDS) and 100 mg/ml proteinase K (Boehringer) which was then allowed to digest for 3 hr at 60° C followed by three extractions with an equal volume of phenol:chloroform (1:1) and ethanol precipitation.

For the isolation of DNA from swinepox, fowlpox, and AmEPV tissue culture cells infected at a multiplicity of 0.1 were collected at 4-6 days postinfection by first scraping the cells into the medium followed by centrifugation at 800 x g for 5 min at 4° C. Each 5×10^{6} of infected cells was then resuspended in 0.5 ml phosphate buffered saline (PBS=10

mM sodium phosphate plus 150 mM NaCl [pH 7.2]) that contained 40 mM EDTA and incubated for 5 min at 37° C. Next 0.5 ml of 1.5% low melt agarose (SeaPlaque, FMC) containing 120 mM EDTA, prewarmed to 42° C, was added to the cells and gently mixed until a uniform suspension was achieved. The suspension was transferred to an agarose plug mold (BioRad), and the agarose allowed to solidify for 15 min at room temperature. The agarose plugs were then removed from the mold and incubated for 12-16 hr at 50° C in plug lysis buffer (1% Sarkosyl, 100 μg/ml proteinase K, 10 mM Tris-HCl [pH 7.5], and 200 mM EDTA). The lysis buffer was then removed and replaced with 5 ml of sterile 0.5 x TBE electrophoresis running buffer (1 x TBE is 89 mM Tris base, 89 mM boric acid, 1 mM EDTA) and equilibrated at 4° C for 6 hr with 3 changes of 0.5 x TBE buffer. The viral DNA was separated from the cellular RNA and DNA contained within the agarose plug by electrophoresis of the plug using the CHEF (Bio-Rad) pulsed field system. Samples were loaded into a continuous well of a 15 cm 1% agarose gel (SeaKem LE, FMC) and electrophoresis was for 20 hr at 180 V with a continuous ramp of 50-90 sec and performed at 15° C in 0.5 x TBE. The isolated band of viral DNA was visualized by staining with ethidium bromide (0.5 μg/ml), excised from the gel and electroeluted from the agarose by electrophoresis in 0.5 x TBE for 2 hr at 100 V in dialysis tubing followed by a 30 sec reverse pulse. The electroeluted viral DNA was ethanol

precipitated and resuspended in TE (50 mM Tris-HCl [pH 8.0], 1 mM EDTA) for analysis. Approximately 50 μg of SPV viral DNA can be obtained from 10^7 cells by this method.

DNA Electrophoresis, Blotting and Hybridization

The preparation of blots for the terminal fragment analyses and the Southern cross-hybridization analysis involved the digestion of purified viral DNA with the appropriate restriction endonucleases and electrophoresis in a 22 cm, 0.8% agarose gel for 800 V-hr in Tris-phosphate buffer (30 mM sodium phosphate monobasic, 36 mM Tris base, 1 mM EDTA [pH 7.7]). The DNA was transferred to a nylon membrane (Hybond-N, 0.45 micron, Amersham) by a modified Southern blotting protocol (Southern, 1975) involving acid hydrolysis and alkaline denaturation of the DNA. Briefly, the gels were soaked for 2 x 15 min in hydrloysis buffer (0.25 M HCl), 2 x 15 min in denaturation buffer (0.5 M NaOH, 1.5 M NaCl), and finally, 2 x 15 min in neutralization buffer (0.5 M Tris-HCl [pH 7.2], 1.5 M NaCl). Capillary transfer was for 16 hr with low ionic strength phosphate buffer (25 mM sodium phosphate [pH 6.5]). The blots were air dried and UV irradiated with 120 millijoules using the UV Stratalinker 1800 (Stratagene) to fix the DNA.

DNA probes were made by radiolabeling the DNA with [^{32}P]dCTP (3000 Ci/mmol, Amersham) to a specific activity of greater than 10^9 using the random oligo primer extension

method of Feinberg & Vogelstein (1983). Prehybridizations were at 63° C for 1hr with blotto (non-fat dry milk, Carnation) (Johnson et al., 1984) in 6 x SSC, and hybridization was carried out for 16 hr at the same temperature in the same solution. Washes were with 2 x SSC, 0.1% SDS for 2 x 15 min at room temperature followed by 2 x 15 min at 63° C. The blots were then exposed to Kodak X-Omat AR film at -70° C.

For experiments designed to measure the accumulation of viral DNA, electrophoresis was performed on the CHEF apparatus and involved the suspension of total infected cell samples in agarose plugs (approximately 10^6 cells/200 μl plug) as previously described for SPV DNA purification. Southern blotting, probe synthesis, hybridization and autoradiography were the same as described above with the temperature of the hybridization and the last set of washes at 63° C.

The pulsed field electrophoresis used to determine the size of the swinepox virus genome utilized the same electrophoretic conditions as described above for the isolation of swinepox virus DNA from infected tissue culture cells. The DNA was subsequently stained with ethidium bromide and the gel photographed.

Terminal Fragment Analysis

The identification of the restriction fragments resulting from a BglI digest of SPV DNA that contain the

terminal hairpins of the SPV genome employed the "snapback" method of analysis as previously described (Esposito et al., 1981; Wills et al., 1983). Following digestion, the resulting fragments were subject to heat denaturation (95° C for 5 min) followed immediately by rapid cooling in an ice water bath. The fragments were separated by pulsed field electrophoresis for 16 hr at 150 V with a continuous ramp from 50 to 90 seconds followed by 6 hr at 180 V with a continuous ramp from 1 to 4 seconds.

The examination of the terminal heterogeneity involved first the restriction enzyme digestion of genomic DNA. The restriction fragments were then end labeled by a "filling in" reaction using [^{32}P]dCTP (3000 Ci/mmol, Amersham) and the large fragment of E. coli DNA polymerase (New England Biolabs) as described by Maniatis et al. (1982). After labeling, fragments were electrophoretically separated on standard 0.8% agarose gels as described above. The gels were collapsed onto slab gel drying paper (Bio-Rad) by subjecting the gels to 2 hr under vacuum without heat using a slab gel dryer (Hoefer Scientific Instruments, Model SE1160). The collapsed gels were then subjected to autoradiography at -70° C.

The analysis of the length of the inverted terminal repeats involved the preparation of a Southern blot containing restriction enzyme HaeII digested genomic swinepox virus DNA. The DNA was restriction digested, loaded into a 4 cm continuous well, and electrophoresed for 150 V-

hr through a 10 cm 0.8% agarose gel (SeaKem LE, FMC). The DNA was transferred to a nylon membrane (Hybond-N, 0.45 micron, Amersham) as previously described. The membrane was cut into 0.5 cm wide strips and hybridized individually to specific probes from near the the left terminus of the SPV genome. The probes were derived from larger cloned DNA fragments that were digested with appropriate restriction enzymes and separated electrophoretically for 150 V-hr in a 10 cm low melting point 0.8% agarose gel (SeaPlaque GTG, FMC) using 1 x Tris acetate buffer containing 0.1 mM EDTA. The subfragments of interest were cut out of the gel, melted at 95° C for 10 min, and [^{32}P]dCTP (3000 Ci/mmol, Amersham) radiolabeled using a modification of the technique described by Feinberg and Vogelstein (1983). The melted gel slices were maintained at 42° C to prevent solidification of the agarose and 32 μl of the slice combined with a mixture, prewarmed to 37° C, containing 5 μl [^{32}P]dCTP (10 μCi/ul, 3000 Ci/mmol, Amersham), 2 μl of 10 mg/ml BSA, 1 μl of DNA polymerase (50 U/μl, New England Biolabs), and 10 μl of oligonucleotide labeling buffer (250 mM Tris HCl [pH 7.6]; 1 M HEPES [pH 6.6]; 28 mM magnesium chloride; 0.3 mM EDTA; 86 μM dATP, dGTP, dTTP; 48 mM mercaptoethanol; 27 A_{260} O.D.U. random sixmer oligonucleotide mix [pd(n$_6$) Amersham]). The labeling reaction (total volume=50 μl) was at 37° C for 1 hr. The blots were prehybridized for 1 hr at 40° C in approximately 7 ml of solution containing 40% formamide, 2.5 x Denhardt's (100 x Denhardt's=2% [w/v] BSA, 2% [w/v]

Ficoll, 2% [w/v] polyvinyl pyrrolidone), 6 x SSPE (20 x SSPE=3.6 M NaCl, 0.2 M sodium phosphate, 0.02 M EDTA), 0.1% SDS, and 2.5 mg denatured salmon sperm DNA. The radiolabeled probes were denatured by heating to 95° C for 5 min, quick cooled, and then added directly to the prehybridization solution. Hybridization was for 16 hr at 40° C. The blots were washed individually with 50 ml of 2 x SSPE, 0.1% SDS for 2 x 15 min at room temperature, dried, and exposed to film.

Radiolabeling and Resolution of Protein from Virus-Infected Cells

Confluent monolayers of PK-15 cells were infected with swinepox virus or vaccinia virus at a multiplicity of 5. Adsorption was for 2 hr at 37° C in the normal growth media previously described but lacking FBS. After adsorption, the infected cells were maintained in normal growth media that contained 10% FBS. Radiolabeling was performed in media lacking methionine for 1 hr with [^{35}S]methionine (1000 Ci/mmol, Amersham) using 60 μCi per 5 x 10^6 cells. All samples were collected immediately after labeling. The samples used for total protein analysis were harvested by scraping the cells into the medium and pelleting them at 800 x g. The pellet was suspended in 1 ml of PBS, pelleted again, and the supernatant removed. The cell pellet was then lysed by the addition of 100 ul of sample buffer (0.2% SDS,

5% glycerol, 0.2% ME, 15% urea, 50 mM EDTA and 80 mM Tris HCl [pH 8.7]) and stored at -70° C prior to use.

Radiolabeled proteins were resolved by electrophoresis on 10% SDS polyacrylamide (30:1 acrylamide/bis ratio) gels. Samples were subjected to electrophoresis at 70 V for 16 hr in Tris-glycine buffer (25 mM Tris base, 200mM glycine, 0.1 % [w/v] SDS). Gels were processed for fluorography as described by Bonner and Laskey (1974), dried onto filter paper (BioRad, slab gel drying paper), and exposed to film at -70° C.

The immunoprecipitations involved the radiolabeling of uninfected or virus infected cells as described above. Immediately after radiolabeling, the cells were scraped into the medium, chilled, pelleted by centrifugation at 800 x g for 5 min at 4° C, and resuspended in 0.5 to 1.0 ml of RIPA buffer (0.15 mM NaCl, 1% SDS, 1% Triton X-100, 0.1% sodium deoxycholate, 10mM Tris hydrochloride [pH 7.4], 100,000 U of aprotinin per ml) plus 1 mM phenylmethylsulfonyl fluoride. The cells wre incubated at 4° C for 30 min and then sonicated briefly. All subsequent steps were also performed at 4° C. Cellular debris was pelleted by centrifugation at 12,800 x g for 10 min. The supernatant was removed and stored at -70° C prior to immunoprecipitation. Equal amounts (100 μl) of supernatant and NET-NP40 (0.5% NP40, 150 mM NaCl, 5 mM EDTA, 50 mM Tris hydrochloride [pH 7.4] were combined and added to 50 μl of a 10% suspension of Staphylococcus aureus (Cowen strain) which had been heat

killed, fixed, and stored as a 10% solution in NET-NP40. Each sample was mixed, incubated for 15 min, mixed again, and incubated for an additional 15 min. Samples were then centrifuged for 3 min at 10,000 x g to remove proteins adhering nonspecifically to the bacterial surface. The supernatant was removed and mixed with 3 μl of undiluted polyclonal rabbit anti-vaccinia (ATCC) or swine anti-SPV, obtained from swine infected experimentally by scarification, and incubated for 12-15 hr. Samples were then mixed with 50 μl of the S. aureus suspension, incubated for 15 min, mixed, and incubated an additional 15 min. The bacterial pellets were collected by centrifugation at 10,000 x g for 1.3 min. The supernatant was discarded and the pellet was suspended in 800 μl of NET-NP40. The suspension was pelleted two additional times in order to minimize nonspecific adherence of protein. The final pellet was suspended in 50 μl of immunoprecipitation lysis buffer (2% SDS, 30 mM Tris hydrochloride [pH 6.8], 1.5% DTT, 20% glycerol, and 0.05% bromphenol blue) by water bath sonication. The samples were then heated to 100° C for 2 min and cooled to room temperature. Bacterial debris was pelleted by centrifugation at 10,000 x g for 3 min, and the resulting sample supernatants were analyzed by polyacrylamide gel electrophoresis as described previously.

RNA Isolation and Northern Analysis

Total RNA was isolated from uninfected PK-15 cells or cells infected with swinepox virus at a multiplicity of 5 by the guanidinium isothiocyanate/cesium chloride method (Glisin et al., 1974; Chirgwin et al., 1979). The poly (A^+) mRNA was isolated by oligo dT affinity chromatography (Aviv and Leder, 1972). The resultant mRNA was resolved by electrophoresis on a 1.4% formaldehyde/agarose gel for 800 V-hr at room temperature (Lehrach et al., 1977). Capillary transfer to nitrocellulose (Schleicher and Schuell) was for 16 hr with 20 X SSC, and the membrane was baked for 2 hr at 80° C under vacuum. Prehybridization was for 4 hr at 40° C in 50% deionized formamide, 6 x SSPE (20 x SSPE=3.6 M NaCl, 0.2 M sodium phosphate, 0.02 M EDTA), 5 x Denhardt's (100 x Denhardt's=2% [w/v] BSA, 2% [w/v] Ficoll, 2% [w/v] polyvinyl pyrrolidone) and 0.1% SDS with 2.5 mg denatured salmon sperm DNA in a final volume of 50 ml. The probe was radiolabeled with [^{32}P]dCTP (3000 Ci/mmol, Amersham) by the random primer oligo extension method (Feinberg and Vogelstein, 1983). Hybridization was at 40° C for 16 hr in 50 ml of the prehybridization solution. The hybridized blot was washed for 2 x 15 min at room temperature in 2 x SSC, 0.1% SDS and autoradiography was as described previously for the Southern blots.

DNA Cloning and Sequence Analysis

Cloning of the regions of the SPV genome into bacterial plasmid vectors involved the digestion of the viral DNA into easily cloned fragments (<8 kilobase) with appropriate restriction enzymes, and subsequent ligation into the pBlueScript KS+ phagemid (Stratagene). All ligations, including those into M13, were done at 15° C for 12-18 hr.

Each restriction fragment analyzed was sequenced individually. The alignment of adjacent clones, when in doubt, was confirmed by polymerase chain reaction using primers that corresponded to the ends of the individual clones. The entire sequencing project was accomplished by a combination single stranded M13 based dideoxy-sequencing and double stranded plasmid based dideoxy-sequencing in order to maximize the accuracy of the sequence, while minimizing the time required.

Beginning with a single SPV fragment cloned into the BlueScript KS+ vector, M13 based sequencing was used to generate the majority of the sequence data. The M13 clones were generated from the cloned SPV fragments by the following protocol. Individual SPV fragments were sheared randomly by sonication, and the 400-600 bp fragments were separated by gel electrophoresis and extracted from the gel by the freeze squeeze technique. The fragments were then "blunt-ended" with T4 DNA polymerase (International Biotechnology Incorporated) and ligated into the SmaI site of the double-stranded replicative form of M13mp19 (Messing

et al., 1977; Messing and Vieira, 1982). The ligation products were transformed into E. coli (strain UT 481), made competent by the rubidium chloride method (Kushner, 1978), and grown for 12-16 hr on nonselective media (2 x YT). Plaques were tested by hybridization to the parent SPV clone, and positive plaques were picked individually and grown for 7-14 hr in 1.5 ml of 2 x YT with a 1:100 diluted inoculum of a log phase culture of UT 481 cells. Bacteria were then pelleted by centrifugation (5 min at 12,800 x g), and the supernatant, containing the single stranded infectious M13 clones, was removed and stored at 4° C. A second growth phase, overnight in 10 ml of 2 x YT, with an inoculum of 100 μl of the M13 supernatant and 40 μl of log phase bacteria, was then used to amplify the clones. The bacteria were pelleted, as before, and 8 ml of the supernatant was removed. The phage were precipitated from the supernatant by a 20 min incubation at room temperature following the addition of 2 ml of NaCl-PEG (2.5 M NaCl, 20% [w/v] PEG-8000). The precipitated M13 phage clones were pelleted by centrifugation for 10 min at 8000 x g, resuspended in 300 μl TE (50 mM Tris, 1 mM EDTA [pH 8.0]), and extracted one time with an equal volume of phenol and then once with an equal volume of chloroform. The DNA was ethanol precipitated (5 min at -70° C), pelleted (15 min at 12,800 x g), and resuspended in 40 μl of TE. Exactly 2 μl of this resuspended M13 clone was then used as the template for each sequencing reaction. The individual clones were

sequenced from these single-stranded clones utilizing a single universal primer (New England Biolabs #1212) located near the cloning site. All sequencing reactions employed the dideoxynucleotide chain termination method (Sanger et al., 1977; Sanger et al., 1980), using a modified T7 polymerase (Tabor and Richardson, 1987)(Sequenase, Promega), and utilized the incorporation of [^{35}S]dATP, as described in the manufacturer's DNA sequencing instruction manual (United States Biochemical Corpoation). Sequencing reaction products were separated by electrophoresis for 42,000 V-hr on 8% polyacrylamide gels (83 cm length; 0.4 mM thick) containing 11.2 M urea, using the BRL (Bethesda Research Laboratory) model S1 sequencing gel apparatus, and a Bio-Rad model 3000Xi power supply. Following electrophoresis, the gels were soaked for 15 min in fixing solution (5% methanol; 5% acetic acid), and dried onto filter paper (Whatman 3 MM) under vaccuum for 40 min at 70° C. The dried gels were subjected to autoradiography at -70° C.

The DNA sequences obtained for individual clones were manually entered into a MacIntosh IIci computer using the DNA Inspector II+ program. The files were subsequently transferred to a DEC VAX, the DNA sequence analyzed, and contiguous sequences aligned using the program of Staden (1986). After completion of approximately 90-95% coverage for a given fragment, sequencing directly from the SPV fragment cloned in the plasmid vector was utilized. This required the synthesis of specific primers adjacent to the

gaps present in the sequence, and utilized the same sequencing protocol as used for the M13 sequencing. However, the double stranded plasmid DNA required denaturation (in 0.2 M sodium hydroxide for 5 min at room temperature) and ethanol precipitation (with 1.5 M ammonium acetate) prior to the annealing reaction. The use of plasmid sequencing allowed for the rapid and specific compilation of the remaining sequence for the gaps within the M13 derived data.

The University of Wisconsin Genetics Computer Group programs (Devereux et al., 1984) were used to analyze the completed DNA sequence data for putative open reading frames, and detect the homology of SPV open reading frames to other sequences in the genbank database. Specifically, TFASTA (Pearson and Lipman, 1988) was used to compare each SPV open reading frame to the genbank database, and identify homology to existing proteins. The ALOM program (Klein et al., 1985), of the MIT DNA analysis package, was used to detect potential membrane spanning domains. The MacPattern program (Fuchs, 1990) was used to identify potential protein modification sites.

CHAPTER 3

COMPARATIVE ANALYSIS OF SPV VERSUS OTHER POXVIRUSES

The poxviruses are an extremely diverse family of viruses. The proper classification of individual members is a subject of debate as new members of the family are discovered. The classification of a novel virus into the Poxviridae family is based on several characteristic features common among all members including the morphology of the virion and the cytoplasmic location of the entire viral lifecycle (Matthews, 1982). The family is divided into two subfamilies, the Chordopoxvirinae and the Entomopoxvirinae, based on host range; the Chordopoxvirinae containing poxviruses capable of infecting vertebrate hosts and the Entomopoxvirinae those that infect invertebrate hosts. There is no known poxvirus that is capable of infecting both a vertebrate and invertebrate host. The Chordopoxvirinae subfamily is further subdivided into numerous genera based on antibody neutralization data. Namely, that an antiserum directed against one member of a given genus will neutralize the infectivity of all other members of that same genus, but will not neutralize members of other genera. This is the basis for classifying SPV into its own separate genus, Suispoxvirus. However, the data in

the literature regarding SPV conclude that SPV infections induce no neutralizing antibodies (Shope, 1940; Schwarte and Biester, 1941). Therefore, this classification based on a neutralization assay would seem rather tenuous. Additional support for the uniqueness of SPV includes several features noted in natural infections including limited pathogenesis, restricted host range, and the extended duration before clearing of the virus (Datt, 1964; Cheville, 1966; Conroy and Meyer, 1971; Kasza et al., 1960; Kasza and Griesemer, 1962; Shope, 1940). However, considering all the evidence, there clearly existed the possibility that SPV was merely an attenuated version of some other poxvirus, such as vaccinia, for which attenuated deletion mutants have been described that exhibit limited host range and pathogenesis (Perkus et al., 1990a). Therefore, several experiments were designed to test the relatedness of SPV to other poxviruses, and to vaccinia in particular. These incuded assays at the DNA level by DNA cross-hybridizations, and at the level of immuno-crossreactivity via immunoprecipitations.

The Cross-hybridization of SPV DNA to the DNA of Other Poxviruses

In order to assess the degree of relatedness at the DNA level of SPV to other poxviruses, we have assayed the ability of radiolabeled SPV DNA to hybridize at low stringency to the DNA of vaccinia, shope fibroma, fowlpox

and entomopoxviruses. The results of this experiment are shown in Figure 3-1, Panel A in which a blot containing restriction enzyme digests of the DNA from fowlpox virus (lane 1), entomopoxvirus (lane 2), SPV (lane 3), Shope fibroma virus (lane 4), and vaccinia virus (Lane 5) was hybridized to radiolabeled SPV DNA. The SPV DNA probe did not hybridize to the DNA of any of the other poxviruses and, therefore, SPV DNA appears to lack, at this level, any significant degree of homology to the other poxviruses examined. This conclusion is consistent with the dissimilarity of the HindIII digest pattern of SPV DNA as compared to the pattern of the HindIII digests of the other poxviruses (Figure 3-1, Panel B).

Immunoprecipitation of SPV and Vaccinia Proteins With Homologous and Heterologous Antisera

The previous classification of swinepox virus into a genus distinct from all other poxviruses was based primarily on the lack of crossreactive neutralizing antibodies. However, this assay is quite limited and would not detect any crossreactivity between non-neutralizing epitopes. Therefore, we readdressed the issue of relatedness through immuno-crossreactivity using immunoprecipitations of proteins isolated from infected cells to explore the antigenic relatedness of SPV and vaccinia. The results of this experiment are shown in Figure 3-2. Lanes A include

Figure 3-1. Cross hybridization of swinepox virus DNA to other poxvirus DNA's. Approximately 500 ng of purified viral DNA of fowlpox virus (lanes 1), entomopox virus (lanes 2), SPV (lanes 3), Shope fibroma virus (lanes 4) and vaccinia virus (lanes 5) was digested with HindIII, separated electrophoretically and Southern blotted as described in Chapter 2. Hybridization was at 50° C with a SPV specific radiolabeled probe in panel A. Panel B shows the same blot hybridized at high stringency (63° C) to a probe derived from the combined DNA of all 5 viruses in order to show all the viral DNA bands which result on HindIII digestion. Lambda HindIII marker positions are indicated in kb.

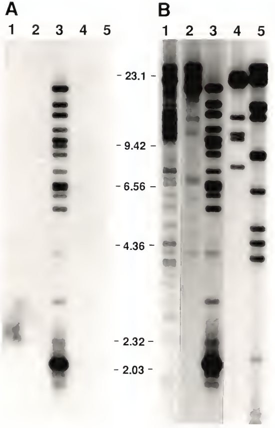
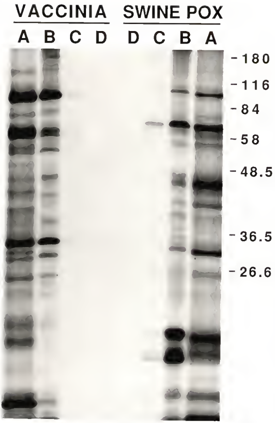

A
1 2 3 4 5
B
1 2 3 4 5
23.1
9.42
6.56
4.36
2.32
2.03

Figure 3-2. Swinepox virus immunologic cross reactivity with vaccinia virus. The cross reactivity of anti-vaccinia and anti-swinepox virus sera was analyzed by immunoprecipitation. Radiolabeled uninfected, vaccinia infected, or SPV infected PK-15 cells were separated on a denaturing 10% polyacrylamide gel. Infected samples were not precipitated (lanes A), immunoprecipitated with homologous antiserum (lanes B), or immunoprecipitated with heterologous antiserum (lanes C). Immunoprecipitations of uninfected PK-15 cells with each antiserum are shown in lanes D. Molecular weight standards are indicated in kD.


VACCINIA
SWINE POX
A B C D
D C B A
-180
-116
-84
-58
-48.5
-36.5
-26.6

total radiolabeled protein samples and are not immunoprecipitations. Lanes B are immunoprecipitations using the appropriate homologous antisera and demonstrate the quality of the two antisera as well as the large number of immunogenic, immunoprecipitable peptides. This is in contrast to lanes C in which very few proteins are precipitated by the heterologous antisera. From this data, I conclude that there are few, if any, shared epitopes between these poxviruses indicating they are antigenically unrelated.

CHAPTER 4

THE SWINEPOX VIRUS INFECTIOUS CYCLE

The literature concerning natural and experimental infections of swine with SPV describes a lingering infection with persistance of the virus in host tissues until nearly 4 weeks postinfection (Shope, 1940). The normal clearance time for an orthopoxvirus infection as described by Fenner et al.(1989) is 10-14 days. This discepancy could be attributable to several factors including the effectiveness of the immune response of the host and the kinetics of expression of the virus itself. There is much information in the literature regarding the kinetics of gene expression during vaccinia virus growth in tissue culture cells (Moss, 1990). The entire vaccinia life cycle, including early and late gene expression, DNA replication, and virion morphogenesis occurs rapidly with mature infective viral progeny produced within 24 hr. However, there has been little experimental data accumulated regarding the repicative cycle of poxviruses other than vaccinia. During experiments involving the initial characterization of SPV it soon became obvious that the rate of SPV development was quite different than that of vaccinia. Therefore, experiments were designed to more closely examine the

kinetics of SPV gene expression in tissue culture compared to vaccinia virus.

Swinepox Virus Growth in Tissue Culture

Swine kidney (PK-15) cells are permissive for the productive infection of either swinepox virus (Garg and Meyer, 1972) or vaccinia virus. However, the cytopathology of infection differs dramatically for these two viruses as shown in Figure 4-1. Noticeable cytopathic effect (CPE) is evident in cells infected with vaccinia by 1 day postinfection (Panel A); however, CPE is not noticeable in cells infected with SPV until 4 days postinfection (Panel I). In the case of vaccinia infected cells, once CPE is noted, spread of the virus and destruction of the surrounding monolayer (Panels B & C) rapidly ensues. By contrast, the SPV cytopathology remains localized and is mostly limited to self-contained foci with little immediate effect on the surrounding monolayer (Panels I & J).

Kinetics of Vaccinia and SPV DNA Accumulation

The accumulation of SPV or vaccinia DNA within infected cells was analyzed by a combination of pulsed field electrophoresis and Southern blot techniques. The rationale for this experiment assumes that once DNA replication is initiated the DNA will accumulate in the infected cell and eventually become detectable above the background level. The

Figure 4-1. Swinepox virus growth in tissue culture. Comparative analysis of vaccinia virus and swinepox virus growth in tissue culture cells. Confluent monolayers of porcine kidney (PK-15) cells were infected with either vaccinia virus (strain IHD-W) or SPV at a multiplicity of 1×10^{-4}. Adsorption was for 2 hr in media without serum. The inoculum was then replaced with media containing 10% FBS and dishes were incubated undisturbed at 37° until photographed. Panels A, B and C are vaccinia infected cells at 1, 2 and 3 days post-infection, respectively. Panels D (day 1) and E (day 5) are uninfected cells used as a control. Panels F through J represent SPV infected cells at days 1 through 5, respectively.

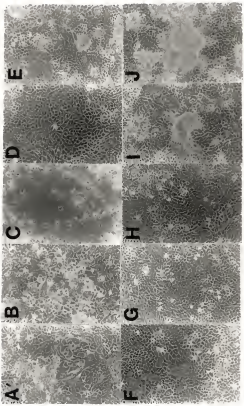
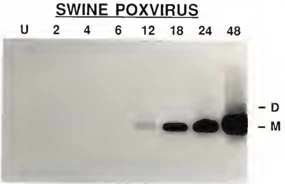
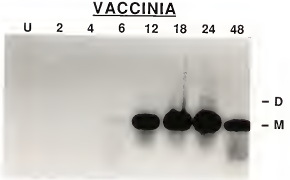

A
B
C
D
E
F
G
H
I
J

Figure 4-2. Analysis of vaccinia virus and swinepox virus DNA accumulation kinetics. Uninfected PK-15 cells, or cells infected with either vaccinia or SPV at a MOI of 5 were collected, lysed and then the viral DNA separated by pulsed field electrophoresis. Southern blotting and hybridizations were as described in Chapter 2. The time postinfection is indicated above each lane. The lane labeled U is an uninfected cell sample. The migration position of viral DNA monomers (M) and dimers (D) is indicated.


SWINE POXVIRUS
U 2 4 6 12 18 24 48
- D
- M


VACCINIA
U 2 4 6 12 18 24 48
- D
- M

results of this analysis for SPV and vaccinia are shown in Figure 4-2. Swinepox virus DNA was not detected until 12 hr postinfection. In additional experiments in which we examined, in detail, time intervals between 6 and 12 hr postinfection, we have been unable to detect SPV DNA earlier than 11 hr postinfection (data not shown). These results are in contrast to what is observed for vaccinia infections where DNA accumulation is readily detectable by 6 hr postinfection. SPV and vaccinia also differ as to when DNA accumulation becomes maximal. The level of vaccinia DNA peaks by 24 hr postinfection and actually decreases by 48 hr postinfection. SPV DNA, on the other hand, continues to accumulate throughout the 48 hr time period examined in this experiment.

Kinetics of Swinepox Virus Protein Expression

SPV protein synthesis was examined by the radiolabeling of the newly synthesized protein within cells infected with SPV at various times postinfection followed by an analysis of the products by SDS-polyacrylamide gel electrophoresis, either with or without prior immunoprecipitation. The total radiolabeled protein pattern of SPV infected cells is shown in Figure 4-3. Little or no virus specific proteins were detectable prior to 24 hr postinfection. However, between 24 to 48 hr postinfection, viral proteins became clearly evident and were synthesized in ever increasing amounts with

Figure 4-3. Kinetics of total protein expression in swinepox virus infected tissue culture cells. PK-15 cells infected with SPV at a moi=5 were radiolabeled for 1 hr prior to collection and harvested as described in Chapter 2. Proteins were separated on denaturing 10% polyacrylamide gels. The time of sample collection is indicated above each lane. The lane labeled E represents a sample maintained in 40 μg/ml cytosine arabinoside, an inhibitor of DNA replication, which blocks late but not early viral protein synthesis. Lane U represents an uninfected control sample. Molecular weights are indicated in kD.

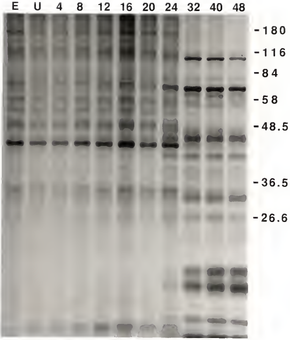
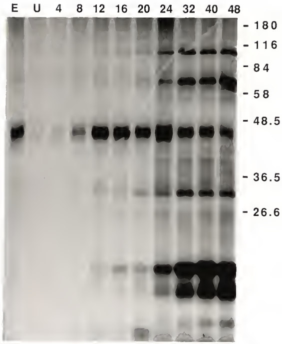

E U 4 8 12 16 20 24 32 40 48
-180
-116
-84
-58
-48.5
-36.5
-26.6

Figure 4-4. Analysis of swinepox virus protein synthesis by immunoprecipitation with anti-SPV sera. Infections, protein labeling, collection of samples, and immunoprecipitations are described in Figure 4-3 and Chapter 2. The time of sample collection is indicated above each lane. The lane labeled E represents a sample infected in the presence of 40 μg/ml cytosine arabinoside, and lane U is an uninfected control. Molecular weights are indicated in kD.

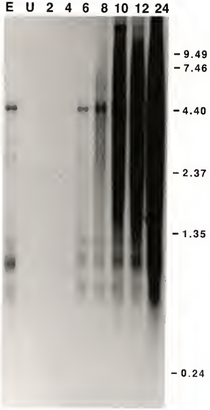

E U 4 8 12 16 20 24 32 40 48
- 180
- 116
- 84
- 58
- 48.5
- 36.5
- 26.6

time. It is also interesting to note that host protein synthesis continues until between 24 and 32 hr postinfection. Vaccinia virus, by contrast, inhibits host protein synthesis much more rapidly. The DNA accumulation experiments previously described revealed SPV DNA replication had begun by 12 hr postinfection. It was, therefore, somewhat surprising that viral protein synthesis was not apparent until 24 hr postinfection. Therefore, immunoprecipitation of viral proteins was utilized as a more sensitive and specific method for the detection of SPV protein synthesis (Figure 4-4). This experiment allowed us to detect SPV protein synthesis as early as 4 hr postinfection, and in agreement with the DNA accumulation experiment, late viral proteins were evident by 12 hr postinfection. The lane labeled E in Figures 4-3 and 4-4 represents a sample of cells infected in the presence of cytosine arabinoside, an inhibitor of DNA replication that prevents subsequent late protein synthesis. Therefore, the samples in these lanes should contain only early viral proteins. In Figure 4-4 (Lane E), a major immunoprecipitable early viral protein of approximately 46 kD is clearly evident under conditions (+ CAR) where unregulated early expression leads to extended synthesis of early proteins. This protein can also be detected in a normal infection by 4 hr if the autoradiograms are overexposed.

Kinetics of Swinepox Virus mRNA Synthesis

Evidence based on our experience with vaccinia as well as other poxviruses has clearly demonstrated that early mRNAs are synthesized as discrete entities of a unique size, as opposed to the majority of late mRNAs which are polydisperse (Cooper et al., 1981). One can, therefore, estimate the initiation of late mRNA synthesis as the time at which the mRNA encoded within a given DNA fragment becomes ill-defined, i.e., polydisperse. The kinetics of SPV mRNA synthesis for one region of the viral genome has been examined by Northern blot analysis and the data are shown in Figure 4-5. Cycloheximide, an inhibitor of protein synthesis, is commonly used in poxvirus infections to accentuate and prolong the synthesis of early mRNAs (Woodson, 1967). Under these conditions, no late messages are expressed, presumably because the translation of early proteins required for DNA synthesis and expression of late genes is blocked through the action of the drug. Therefore, the lane labeled E, in which RNA was isolated and examined from cells infected in the presence of cycloheximide, represents the expression of only early viral mRNAs. The data show that early SPV mRNA can be detected as early as 4 hr postinfection and that late gene expression was initiated at 8-10 hr postinfection at which time the RNA pattern becomes heterodisperse.

Figure 4-5. Kinetics of swinepox virus RNA synthesis. Poly (A) mRNA isolated from SPV infected PK-15 cells was separated on a 1.4% formaldehyde/agarose gel, tranferred to nylon membrane, and hybridized as described in Chapter 2. The probe was an 8.2 kb EcoRI SPV fragment that spans the junction of SPV HindIII fragments G and F. The time of sample collection is indicated above each lane. Lane U represents an uninfected control sample. Lane E represents a sample maintained in the presence of cycloheximide, an inhibitor of protein synthesis, and therefore contains only early viral mRNA. RNA size standards are indicated in kb.


E U 2 4 6 8 10 12 24
- 9.49
- 7.46
- 4.40
- 2.37
- 1.35
- 0.24

CHAPTER 5

ANALYSIS OF THE SPV GENOME

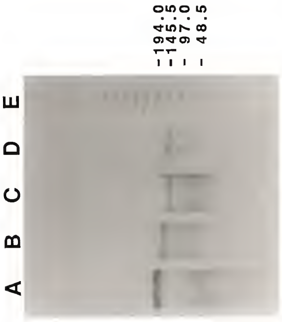
As previously mentioned, the classification of swine poxvirus as a member of the poxvirus family was based on several criteria. First, SPV exhibits the typical virion morphology of a poxvirus, and, second, it appears to replicate exclusively within discrete foci within the cytoplasm of the infected host cell. These are features that all members of the family have in common, however, in addition to these characteristics, more recent studies have demonstrated that all vertebrate poxviruses also seem to share certain common molecular features in terms of their DNA genomes. Among these shared features are the large size of the linear DNA molecules (130-300 kb), the presence of short, single stranded loops of DNA at each end of the genome serving to covalently crosslink the molecule, and the presence of repetitive elements at each end of the molecule in an inverted orientation with respect to each other (Moss, 1990). These repetitive elements, referred to as inverted terminal repeats (ITRs), are of variable length, depending on the species of poxvirus, but are invariably present with the possible exception of smallpox (Esposito and Knight, 1985; Mackett and Archard, 1979). In addition, there are

also a series of small direct repeats near the each terminus of the genome for all members of the Orthopoxvirus genus (Pickup et al.,1982; Wittek and Moss, 1980). These repeats are not present among the leporipoxviruses (Upton et al., 1987b) and there are few data regarding this region of the DNA of the other poxvirus genera. Therefore, there is a substantial amount of data regarding the genomes of poxviruses, some of it specific for the entire family, and some which is genus- and species-specific. The characterization of the genome of SPV was therefore undertaken for several reasons. For one, this information would assist the classification of SPV by identifying family, genus, or species specific traits. It would also provide essential evidence important for furthering our currently limited understanding of the evolutionary relationship between SPV and other members of the poxvirus family. Lastly, these data would allow for the possible identification of the unique features of SPV, thereby adding to our knowledge of poxvirus genome structure-function relationships and of the molecular basis of poxvirus pathogenesis.

Size of SPV Genomic DNA

Comparative pulsed field electrophoresis was used to estimate the size of the swinepox virus genome. As shown in Figure 5-1, SPV DNA (lane B) exhibits an electrophoretic

Figure 5-1. Size of the swinepox virus genome. The size of the swinepox virus genome was determined by pulsed field electrophoresis. DNA extracted from purified virions of vaccinia virus (lane A), swinepox virus (lane B), myxoma virus (lane C) and Shope fibroma virus (lane D) were separated on a 1% agarose gel as described in Chapter 2. Lambda ladder size standards are shown in lane E with the sizes of the first four bands indicated in kilobases.

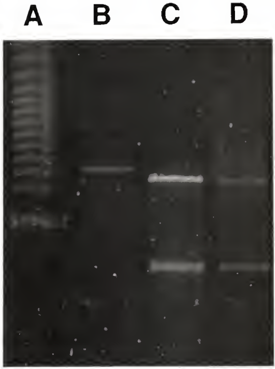

A
B
C
D
E
194.0
145.5
97.0
48.5

mobility between that of the orthopoxvirus vaccinia virus (lane A) and the leporipoxviruses, myxoma (lane C) and Shope fibroma virus (lane D). The size of the vaccinia genome is approximately 185 kb (Dales and Pogo, 1981) and the sizes of myxoma and Shope fibroma virus are estimated to be 163 kb and 160 kb, respectively (Delange et al., 1984; Russell and Robbins, 1989). Therefore, the conclusion from these data is that the size of the SPV genome is approximately 175 kb. This estimate is also in agreement with the size deduced from the lambda ladder size standard shown in lane E.

SPV DNA Terminal Crosslinks

The DNA from all poxviruses examined to date have termini that are covalently closed by a short single stranded loop of DNA (Baroudy et al., 1982). This same characteristic for SPV DNA is demonstrated by a "snap-back" analysis (Esposito et al., 1981; Wills et al., 1983) as shown in Figure 5-2. The enzyme BglI cuts the SPV genome into three fragments of approximately 22, 24 and 105 kb (lane C). Following digestion with BglI, the DNA was heat-denatured and quick chilled. Only terminal fragments which are crosslinked will then spontaneously reanneal. As shown in Lane D, the 24 and 105 kb fragments but not the 22 kb fragment, were able to reanneal (lane D) and remain as discrete bands. Therefore, from these data we conclude that SPV does have covalently closed termini that the 22 kb

Figure 5-2. "Snapback" analysis of the BglI digest of swinepox virus genomic DNA. Purified SPV DNA was separated by pulsed field electrophoresis on a 1% agarose gel as described in Chapter 2. Undigested samples of purified DNA (lane B), or DNA digested with BglI without further treatment (lane C), or digested with BglI and subsequently heat denatured and quick chilled (lane D) prior to electrophoresis are shown in the figure. The lambda ladder size standards are shown in lane A.


A
B
C
D

fragment is the internal fragment. These data were useful for the preliminary mapping of additional digests of the SPV genomic DNA.

Detailed Restriction Mapping of the SPV Genome

A preliminary restriction map of SPV was constructed by doing single and double digests with the restriction endonucleases BglI, XhoI and SalI. XhoI has only a single recognition site in SPV DNA, whereas the other two enzymes each cut SPV DNA twice. The use of pulsed field electrophoresis was critical for this analysis because it enabled the separation, accurate sizing and identification of these large fragments. The restriction endonucleases HindIII, AvaI, HaeII and KpnI were selected for more precise mapping because each cut SPV DNA into between 8 and 16 fragments, most of which were easily separable by standard electrophoretic techniques. The size of each of these fragments was estimated based on the electrophoretic mobility when compared to HindIII single digests and HindIII/Eco RI double digests of lambda DNA as size standards. A tabulation of the size and number of fragments from each digest, as determined from electrophoretic mobility, as well as the size of each fragment derived from calculated values based on the overall size of the SPV genome (Figure 5-1) are shown in Table 5-1. Double digests of SPV using one of the four enzymes which cuts frequently

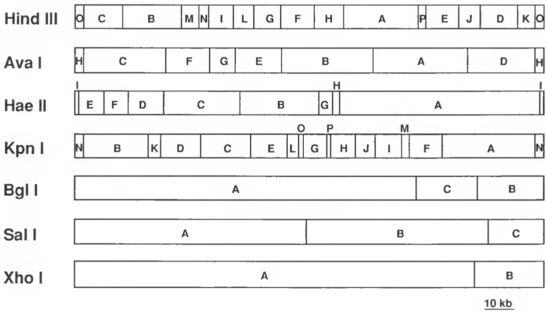


Figure 5-3. Swinepox virus restriction maps. The restriction maps of swinepox virus DNA for the endonucleases Hind III, Ava I, Hae II, Kpn I, Bgl I, Sal I and Xho I are shown. The sizes of the individual restriction fragments are presented in Table 5-1.

Table 5-1. Restriction fragment sizes of the swinepox virus genome.

Fragment	HindIII	AvaI	HaeII	KpnI
A	24.6(28.6)[a]	30.0(34.9)	64.0(74.7)	30.0(35.3)
B	17.7(20.6)	29.5(34.3)	25.8(30.1)	21.0(24.7)
C	14.5(16.9)	28.0(32.6)	24.0(28.0)	15.8(18.6)
D	11.7(13.6)	22.3(25.9)	11.5(13.4)	12.9(15.2)
E	10.4(12.1)	15.1(17.6)	8.1 (9.5)	11.6(13.7)
F	10.4(12.1)	13.3(15.5)	8.0 (9.3)	10.0(11.8)
G	9.6(11.2)	7.9 (9.2)	2.0 (2.3)	8.2 (9.7)
H	8.8(10.2)	2.2 (2.6)x2	1.1 (1.3)x2	8.1 (9.5)
I	7.7 (9.0)			7.9 (9.3)
J	6.9 (8.0)			6.0 (7.1)
K	6.7 (7.8)			4.1 (4.8)
L	6.4 (7.5)			3.3 (3.9)
M	5.6 (6.5)			3.1 (3.6)
N	3.2 (3.7)			2.3 (2.7)x2
O	2.1 (2.4)x2			1.3 (1.5)
P	1.9 (2.2)			0.8 (0.9)
Total	150.3(175)	150.5(175)	149.9(175)	148.7(175)

	BglI	SalI	XhoI
A	[b]104.8(121.9)	[b]69.5(81.9)	[b]126.7(147.3)
B	23.7 (27.6)	[b]58.8(69.3)	24.8 (28.8)
C	21.9 (25.5)	20.2(23.8)	
Total	150.4 (175)	148.5(175)	150.5 (175)

[a] Sizes in parentheses were calculated from the estimated genome size of 175 kilobases. Sizes not in parentheses were determined based on lambda size standards. All sizes are expressed in kilobases.

[b] Size estimated from multiple digests.

in combination with one of the other enzymes which cuts infrequently (BglI, XhoI & SalI) identified the HindIII, AvaI, HaeII and KpnI fragments which overlapped the infrequent cut sites. The maps for these four enzymes were subsequently determined by the hybridization of overlapping fragments as follows.

SPV DNA was digested with HindIII, AvaI, HaeII or KpnI, and the fragments separated electrophoretically in 0.6% agarose gels and Southern blotted as described in Chapter 2. DNA Probes were prepared from individual fragments isolated from identical SPV digests separated by electrophoresis in 0.6% low melting point agarose gels. Each fragment was excised from the gel and radiolabeled in situ by random oligonucleotide extension as described by Feinberg and Vogelstein (1983). The labeled probe fragments were individually hybridized to the blots as described in the Chapter 2. The results of these hybridizations allowed for the identification of overlapping fragments and the construction of the composite restriction maps of SPV DNA as presented in Figure 5-3.

Identification of SPV DNA Terminal Fragments

The terminal fragments of various DNA digests were determined by a novel approach utilizing the analysis of the dimeric DNA intermediates generated during the process of in vivo viral DNA replication (Moyer and Graves, 1981).

Replicative dimers can be isolated from genomic monomers by pulsed field gel electrophoresis (Delange, 1989). This analysis assumes that replicative dimeric forms of SPV DNA, like those of other poxviruses, represent monomers joined in a head-to-head or tail-to-tail fashion. The monomeric and dimeric DNA forms were first resolved from one another, and separately electroeluted. Digestion of the dimeric form and subsequent electrophoretic separation of the resulting fragments should reveal the creation of a new restriction fragment derived from the fused terminal fragments where two monomers are joined in either a head-to-head or tail-to-tail fashion. The corresponding new terminal fusion fragment would be expected to migrate with a molecular weight twice that of the corresponding terminal fragment derived from the monomer. A dimer should contain two termini joined to form a unique fusion fragment plus two terminal fragments as in the original monomer. The data shown in Figure 5-4 demonstrate how this approach was used to identify the terminal fragment(s) of the <u>Hind</u>III digest of SPV DNA. When <u>Hind</u>III digests of monomeric and dimeric DNA are hybridized to total genomic DNA, a unique fragment of 4.2 kb present only in dimers is noted (figure 5-4, Panel B). If one assumes the unique fragment results from head-to-head or tail-to-tail fusion of two monomers, then this unique fragment should be derived from the <u>Hind</u>III O fragment previously determined to be the terminal-most fragment present at each end of the

Figure 5-4. Identification of the terminal *Hind*III fragment of swinepox virus DNA. Fragments derived from *Hind*III digested SPV monomeric (M) or dimeric (D) DNA were separated electrophoretically and Southern blotted as described in Chapter 2. The blot was hybridized with a radiolabeled probe specific for the SPV *Hind*III fragment O (panel A) or a probe for total SPV genomic DNA (panel B). The arrows indicate the unique junction fragment seen only with the dimeric DNA. Lambda DNA size standards are indicated in kilobases.

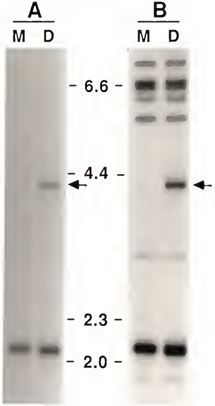
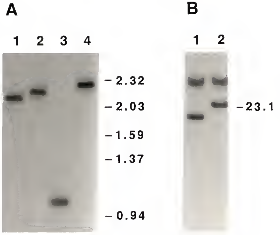

A
M D
B
M D
6.6
4.4
2.3
2.0

Figure 5-5. Identification of the swinepox virus DNA terminal fragments resulting from various digests of SPV DNA. SPV DNA was digested with the appropriate restriction enzyme, electrophoretically separated, Southern blotted, and hybridized with a probe specific for the SPV HindIII terminal fragment (fragment O) as described in Chapter 2. The hybridizations shown in panel A are for SPV DNA digested with HindIII (lane 1), AvaI (lane 2), HaeII (lane 3) and KpnI (lane 4). Panel B contains the SalI (lane 1) and XhoI (lane 2) fragment hybridizations. The position of HindIII and HindIII/EcoRI digested lambda DNA size standards are shown for comparison in kilobases.


A
1 2 3 4
– 2.32
– 2.03
– 1.59
– 1.37
– 0.94
B
1 2
– 23.1

monomers (Table 5-1 and Figure 5-3). The hybridization of HindIII digested monomers and dimers to radiolabeled Hind III O fragment shows this to be the case (Fig. 5-4, Panel A). We conclude that the 2.1 kb fragment (HindIII fragment O) is the terminal-most fragment derived from each end of the HindIII digested SPV genome. The HindIII O terminal fragment was then utilized as a probe to locate the terminal fragments of the other restriction enzyme maps as shown in Figure 5-5. This allowed for the identification of a single terminal fragment of the AvaI digest as fragment H, fragment I of the HaeII digest and fragment N of the KpnI digest with sizes of 2.2 kb, 1.1 kb, and 2.3 kb, respectively (Figure 5-5, Panel A) consistent with cutting within a terminal repetitive element. The hybridization of the 2.1 kb radiolabeled terminal HindIII O fragment to either XhoI or SalI digests of SPV DNA recognizes two bands in each digest (Fig 5-5, panel B). This result together with the results shown in of Fig 5-5, panel A is consistent with the presence of repetitive elements at or near the terminal extremes of the molecule and confirms the previous mapping of the terminal fragments for the XhoI and SalI digests shown in Figure 5-3. The fact that the terminal HindIII fragment detects homologous sequences at both ends of the genome provides evidence that the termini of the swinepox virus genome contain inverted repetitions as has been described for nearly all other poxviruses.

<u>Length of the SPV DNA Inverted Terminal Repeats</u>

The length of poxvirus inverted terminal repeats (ITRs) varies dramatically among the various species of the family. Vaccinia ITRs are larger than 12 kilobases as are those of all members of the leporipox group (Goebel et al., 1990; Upton et al., 1987a). In contrast, smallpox ITRs , if present at all, are estimated to be less than 500 basepairs (Esposito and Knight, 1985; Mackett and Archard, 1979). The length of the SPV ITRs were determined by hybridization analysis as follows. Genomic SPV DNA was digested with the restriction enzyme <u>Hae</u>II, separated electrophoretically, and Southern blotted. <u>Hae</u>II was used because the restriction fragments at each end of the genome just internal to the small 1.1 kb terminal fragment (fragments E and A) are easily separated electrophoretically. Also, the E fragment and the F fragment, which is just internal relative to the entire genome, migrate as a single band. The A and E/F fragments should span the ITRs at alternate ends of the genome. Using small subfragments (2 kb or less) of the E and F fragments as probes, fragments from within the ITR will hybridize to both the E/F and A fragments because of the repetitive nature of genetic content within the ITR. Subfragments not within the ITR will conversely recognize only the E or F fragment from which they were derived. Therefore, the loss of hybridization to the A fragment defines the boundary of the ITR. Figure 5-6 shows the

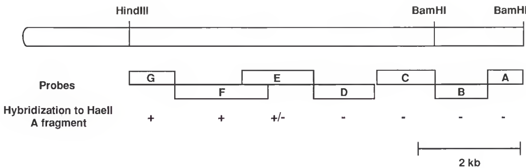
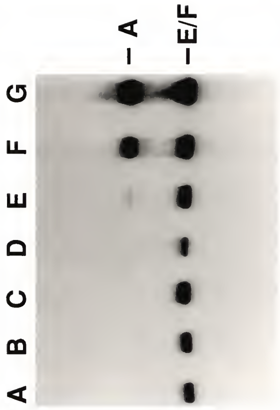


Figure 5-6. Schematic representation of the swinepox virus inverted terminal repeats. Representation includes the probes used, and the results obtained from the analysis of the swinepox virus inverted terminal repeat length. The drawing represents the left terminus of the SPV genome including 7664 bp from the Hind III site at the left end of the Hind III C fragment through the two internal BamHI sites. The relative locations of the individual fragments, designated a through G, used as probes are shown. The length of the fragments are A, 662 bp; B, 1048 bp; C, 1145 bp; D, 1178 bp; E, 1377 bp; F, 1851 bp; and G, 924 bp. The results of the hybridizations shown in Figure 5-7 are also indicated with (+) representing a positive signal, (-) no signal, and (+/-) a weak signal.

Figure 5-7. Length of the swinepox virus inverted terminal repeats. Genomic SPV DNA was digested with HaeII, electrophoretically separated, Southern blotted and hybridized to the various probes shown in Figure 5-6 as described in Chapter 2. The lanes A through G represent the individual fragments that were radiolabeled and used as probes, also described in Chapter 2. The position of the HaeII fragments A and E/F to which the probes hybridized are indicated.

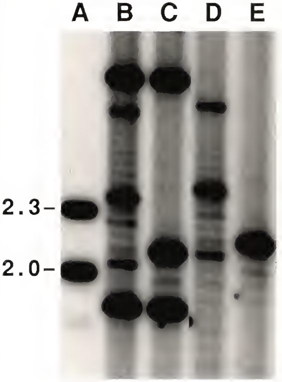

A B C D E F G
A
E/F

derivation of the subfragments used as probes in relation to left end of the SPV genome, the probe designations, and a summary of the hybridization results. Figure 5-7 shows the actual hybridization data showing that probes A through D hybridized only to the E or F fragment while probes E, F, and G hybridized to both the A and E fragments, although the signal with probe E was faint. This evidence limits the ITR boundary to within the region spanned by probe E. Therefore, the length of the SPV ITRs are between 4.3 and 5.6 kb, with the actual length probably closer to the lower limit because of the limited hybridization with the E probe.

Repeat Elements Within the SPV ITRs

When a serially propagated stock of SPV DNA is digested with either *Hin*dIII (Figure 5-8, lane B) or *Ava*I (Figure 5-8, lane D), end labeled, and separated electrophoretically, it is very difficult to discern a single discrete terminal fragment. Instead, a ladder of fragments appear ranging in size from approximately 1.5 kb to 3.0 kb. Each fragment within the ladder is separated by an interval of approximately 70 bp and although each interval is represented, there appears to be several favored forms. However, when the virus is first purified by cloning, a distinct, unique terminal fragment is seen (Figure 5-8, lanes C and E). The simplest interpretation of these data is that the heterogenous population of termini seen are the

Figure 5-8. Characterization of the swinepox virus terminal repeat elements. The DNA from plaque purified (lanes C and E) or serially passed (lanes B and D) SPV was digested with either HindIII (lanes B and C) or AvaI (lanes D and E). Fragments were end labeled and separated electrophoretically as described in Chapter 2. Lane A contains the end labeled HindIII digest fragments of lambda DNA with the size of the 2.32 kb and 2.03 kb fragments indicated.


A
B
C
D
E
2.3–
2.0–

result of multiple recombinatorial events involving a series of repeated elements 70 bp in length. However, the unequivocal confirmation of the presence of these repeats must await detailed sequence analysis.

CHAPTER 6

PARTIAL SPV DNA SEQUENCE ANALYSIS

Studies of poxviruses by DNA sequence analysis, restriction enzyme mapping, and open reading frame analysis have revealed several interesting features concerning poxvirus genome organization including several highly conserved characteristics. The genomes of all members of the family contain a central core element of conserved viral enzymatic and structural genes required for maintenance of a normal poxvirus lifecycle (Hirt et al., 1986; Weir and Moss, 1984; Wittek et al., 1984a; Wittek et al., 1984b). Although the genes in this region are generally not highly conserved at the nucleotide level (except for closely related members such as vaccinia and rabbitpox virus), there is much more conservation at the amino acid level, and the location and orientation of open reading frames is highly conserved (Binns et al., 1988; Drillien et al., 1987; Gershon and Black, 1989). However, the near terminal regions of poxvirus genomes are quite dissimilar among the various species with limited conservation between members of a given genus, but little conservation between genera (Tomley et al., 1988; Upton et al., 1987a). There is evidence that located within these regions is the genetic material responsible for the

wide range of pathogenicity and host range specificity exhibited within the poxvirus family. It is within this region that several host range genes of vaccinia and cowpox have been characterized (Drillien et al., 1981; Gillard et al., 1986; Perkus et al., 1990a). A wide array of gene families with homologies to various host factors have also been identified here. For vaccinia these products include a growth factor, a serine protease inhibitor (SERPIN), and complement component binding analogs, and there has been much speculation regarding the function of these products and how they might contribute to viral pathogenicity in natural infections (Kotwal and Moss, 1988a; Kotwal and Moss, 1989; Stroobant et al.,1985).

This wealth of information regarding vaccinia is in contrast to what is known concerning the genes of SPV. Prior to the studies described herein, SPV had not been characterized at the molecular level. I therefore elected to further our understanding of swinepox gene organization by DNA sequence analysis of selected regions of the genome. I chose to analyze of the region of the genome adjacent to the junction between the inverted terminal repeat at the left hand end of the genome, and a region of internal unique sequences. The rationale for these two choices was that we could gain insight about both the internal conserved region of the virus, and the terminal region of the genome, which in other poxviruses is related to pathogenesis and host

range properties. We hoped to elucidate the reasons for the limited pathogenesis of SPV and its host range restriction to swine by means of this sequence analysis.

Specifically then, sequence analysis of the 2.85 kb internal fragment was chosen to text the hypothesis that the central core of SPV is conserved in terms of open reading frame order, content and orientation, as has been shown for several other poxviruses. This analysis also allows for a comparison of vaccinia intergenic regulatory regions, which are involved in viral mRNA transcript initiation and termination, to the analogous regions in SPV. Although there is not a true vaccinia consensus early or late promotor, there are several features that are conserved among early vaccinia virus genes which are also present and functional in several other poxvirus species and genera. All poxvirus promotor elements appear to be located within approximately 30 nucleotides of the start of transcription and are very A/T rich (Coupar et al., 1987; Mars and Beaud, 1987; Weir and Moss, 1987). Mutagenesis studies have been used to specifically define an early vaccinia promotor consensus sequence AAAAATGAAAAA(T/A)A located 10-15 bp upstream of the transcription initiation site (Davison and Moss, 1990a). Single or multiple nucleotide changes within this sequence usually reduce, but do not eliminate, expression. Since most early vaccinia genes do not conform exactly to the consensus sequence, it has been suggested that variations constitute a

mechanism whereby poxviruses regulate the expression of early genes. Another feature usually present for early transcripts is a conserved TTTTTNT motif (where N represents any nucleotide), that serves as a transcription termination signal, located approximately 20-50 nucleotides upstream of the 3' termination site (Yuen and Moss, 1987).

The examination of the upsteam sequences of numerous late vaccinia genes provided the first evidence for a conserved late promotor element (a TAAAT motif) close to the mRNA start site (Rosel et al., 1986). In fact, the majority of late genes have this motif within the sequence TAAATG, with the ATG serving as the translation initiation codon. Additional mutagenesis studies confirmed the essentiality of this element and further noted runs of A's or T's approximately 20 bp upstream of the start site which contribute to late promotor strength (Davison and Moss, 1989b). The comparison of SPV early and late promotor elements to the vaccinia consensus elements will be useful for determining whether the promotor homologies and ability to cross-function between genera noted among other poxviruses is also true for SPV (Boyle and Coupar, 1986; Boyle and Coupar, 1988; Taylor et al., 1988).

The sequence analysis and subsequent comparison of these regions of the SPV genome and gene products to those of vaccinia and other poxviruses should lead to a better understanding of the phylogenetic relationship between the

members of this viral family. Specific mutagenesis studies would then allow for the identification of specific genetic elements which might be responsible for some of the unique properties of SPV.

Analysis of SPV Conserved Region DNA Sequence

The sequencing of an internal region of the swinepox virus genome was therefore undertaken to determine if the conservation of open reading frames and intergenic regulatory elements noted in other poxviruses was maintained within SPV. This analysis involved the sequencing of 2857 basepairs of DNA from within the SPV HindIII H fragment. The total A+T content of this region was found to be 71.7%. Figure 6-1 represents the relative location of the sequenced region within the SPV genome and the orientation of the open reading frames that were deduced from this sequence. There were 2 complete and one partial open reading frames identified, designated H1L, H2L and H3L, all transcribed towards the left terminal hairpin. The nucleotide sequence of this region and the deduced amino acid sequences are shown in Figure 6-2. Each of the SPV open reading frames corresponded to analogous open reading frames within the HindIII D fragment of vaccinia virus. The gene products, at least in the case of vaccinia, are essential for a productive viral infection. The alignment of the SPV open reading frames to the corresponding ones from vaccinia,

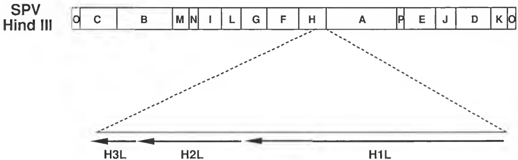


Figure 6-1. Swinepox virus core open reading frames. Schematic representation of the location and orientation of the SPV open reading frames deduced from the conserved region sequence. The Hind III genomic map is shown with an expanded view of the 2.85 kb region at the right end of the Hind III H fragment that was sequenced. The 3 deduced open reading frames designated H1L, H2L, and H3L are indicated.

Figure 6-2. The complete nucleotide sequence and the deduced amino acid sequences from the conserved core of swinepox virus. Nucleotide number 1 begins at the right end of <u>Hind</u>III fragment H and proceeds towards the left terminus. The deduced amino acids are indicated below the first nucleotide of each codon triplet, and the open reading frame designation below the initiating methionine.

```
    AAGCTTTTCATACTTTAAATACGACAATGAATAATACTGTTATTAACTCGATTATAGGTA
  1 ---------+---------+---------+---------+---------+---------+ 60
                               M  N  N  T  V  I  N  S  I  I  G  N
                               H1L
    ATGATGATATTGTTAAACGTCATAATGTATTCGGTGTAGATGTACAAAATCCTACTTTAT
 61 ---------+---------+---------+---------+---------+---------+ 120
       D  D  I  V  K  R  H  N  V  F  G  V  D  V  Q  N  P  T  L  Y

    ATATGCCACAGTATATAACTATAAACGGCATAACCTCTACAGACAGTAACTGCGACCAAC
121 ---------+---------+---------+---------+---------+---------+ 180
       M  P  Q  Y  I  T  I  N  G  I  T  S  T  D  S  N  C  D  Q  H

    ATGTTGTATCTACTTTTGAAATACGTGATCAATATATTACAGCGCTTAGTCATGTTATGC
181 ---------+---------+---------+---------+---------+---------+ 240
       V  V  S  T  F  E  I  R  D  Q  Y  I  T  A  L  S  H  V  M  L

    TAAGCATAGAATTACCAGAAGTTAAAGGTGTTGGTAGATTCGGTTATGTTCCATATGTTG
241 ---------+---------+---------+---------+---------+---------+ 300
       S  I  E  L  P  E  V  K  G  V  G  R  F  G  Y  V  P  Y  V  G

    GATATAAGTGTATTCAACATGTATCTATATCCAGCTATGATGATATATTATGGGAATCAT
301 ---------+---------+---------+---------+---------+---------+ 360
       Y  K  C  I  Q  H  V  S  I  S  S  Y  D  D  I  L  W  E  S  S

    CCGGAGAAGATTTATATAACTCGTGTTTAGATAATGATACGGCATTAACAAATTCTGGAT
361 ---------+---------+---------+---------+---------+---------+ 420
       G  E  D  L  Y  N  S  C  L  D  N  D  T  A  L  T  N  S  G  Y

    ATTCGCATGAACTTAATACAATATCTACAGGATTGACTCCAAACGACACAATTAAAGAAT
421 ---------+---------+---------+---------+---------+---------+ 480
       S  H  E  L  N  T  I  S  T  G  L  T  P  N  D  T  I  K  E  S

    CTACAACTGTGTATGTTTATATAAAAACTCCCTTTGATGTAGAGAAAACATTTAGTAGTT
481 ---------+---------+---------+---------+---------+---------+ 540
       T  T  V  Y  V  Y  I  K  T  P  F  D  V  E  K  T  F  S  S  L

    TAAAGTTGGCAGATACAAAAATTGTCATTACCGTCACATTTAATCCTGTTTCTGATATTA
541 ---------+---------+---------+---------+---------+---------+ 600
       K  L  A  D  T  K  I  V  I  T  V  T  F  N  P  V  S  D  I  I

    TTATAAGAGATATAACGTTTAATTATGATAATTTCGTTAAAGATTTTGTCTATGTTACAG
601 ---------+---------+---------+---------+---------+---------+ 660
       I  R  D  I  T  F  N  Y  D  N  F  V  K  D  F  V  Y  V  T  E

    AACTCAGTTGTATAGGGTATATGGTAAAAAATATACAAATAAAACCGTCTTATATAGAAA
661 ---------+---------+---------+---------+---------+---------+ 720
       L  S  C  I  G  Y  M  V  K  N  I  Q  I  K  P  S  Y  I  E  R

    GGCCTAGAAGAGTATTTGGTCAATTAAATCAATCTACAGCTGTAATATCTGATGTTCATT
721 ---------+---------+---------+---------+---------+---------+ 780
       P  R  R  V  F  G  Q  L  N  Q  S  T  A  V  I  S  D  V  H  S

    CTGTATCATCATTATCTGTATATATCAAACCATACTATGGAAATGCAGATAATAAATTCA
781 ---------+---------+---------+---------+---------+---------+ 840
       V  S  S  L  S  V  Y  I  K  P  Y  Y  G  N  A  D  N  K  F  I
```

```
      TATCATATCCTGGATATTCACAATCAGAAAAAGATTATATATGTGTTTTTGTAGAGAGAC
 841 ---------+---------+---------+---------+---------+---------+ 900
        S  Y  P  G  Y  S  Q  S  E  K  D  Y  I  C  V  F  V  E  R  L

      TTTTAGATGACCTCGTCACAGTATGTGATACATCTCCAAAATGGTTTCCAGAGACTGCAG
 901 ---------+---------+---------+---------+---------+---------+ 960
        L  D  D  L  V  T  V  C  D  T  S  P  K  W  F  P  E  T  A  E

      AACTCGTTGAAGTACCAAATAGTGGTATTGTAACAATACAAGATGTTGATATTTTTGTTC
 961 ---------+---------+---------+---------+---------+---------+ 1020
        L  V  E  V  P  N  S  G  I  V  T  I  Q  D  V  D  I  F  V  R

      GTATAGATAATGTTCCATGTAATATGAAAGTTTATTTTCATACTAATATATTAGTATTTG
1021 ---------+---------+---------+---------+---------+---------+ 1080
        I  D  N  V  P  C  N  M  K  V  Y  F  H  T  N  I  L  V  F  G

      GAACACGAAAAAATTCAGTTACATATAATTTATCTAAAAAGTTTACAACGATAACAGGCA
1081 ---------+---------+---------+---------+---------+---------+ 1140
        T  R  K  N  S  V  T  Y  N  L  S  K  K  F  T  T  I  T  G  T

      CCTATAGCGAAAGCACTAATAGAATTATGTTTTCTCATGTGTCACATTCTATAAATATTA
1141 ---------+---------+---------+---------+---------+---------+ 1200
        Y  S  E  S  T  N  R  I  M  F  S  H  V  S  H  S  I  N  I  T

      CAGATGTATCAATTCCTGTAAGTGTATGGACCTGTCAACGTAATATATACAACGGTGATA
1201 ---------+---------+---------+---------+---------+---------+ 1260
        D  V  S  I  P  V  S  V  W  T  C  Q  R  N  I  Y  N  G  D  N

      ATCGATCAGAATCATCAAAAAATAAAGATTTATTTATTAATGATCCGTTCATAAAAGGTA
1261 ---------+---------+---------+---------+---------+---------+ 1320
        R  S  E  S  S  K  N  K  D  L  F  I  N  D  P  F  I  K  G  I

      TCGATTTCAAAAATAAAACCGATATTATTTCTAGATTAGAAGTAAGATTTGGTAACGATG
1321 ---------+---------+---------+---------+---------+---------+ 1380
        D  F  K  N  K  T  D  I  I  S  R  L  E  V  R  F  G  N  D  V

      TATTATATTCTGAAACGAGTCCTATTTCTAAAGTTTACAATGATCTACTTTCTAATCATA
1381 ---------+---------+---------+---------+---------+---------+ 1440
        L  Y  S  E  T  S  P  I  S  K  V  Y  N  D  L  L  S  N  H  K

      AATGTGGTATGAGAACATTACGATTTAATTTCACACCCCCTACATTTTTTAAACCCACTA
1441 ---------+---------+---------+---------+---------+---------+ 1500
        C  G  M  R  T  L  R  F  N  F  T  P  P  T  F  F  K  P  T  T

      CAATTGTTGCAAATCCTTCTAGAGGTAAGGATAAATTATCCGTACGTGTCGTATTTACCT
1501 ---------+---------+---------+---------+---------+---------+ 1560
        I  V  A  N  P  S  R  G  K  D  K  L  S  V  R  V  V  F  T  S

      CGTTAGATCCTAATAATCCTATCTATTACATATCGAAACAATTGGTATTAGTTTGTAAAG
1561 ---------+---------+---------+---------+---------+---------+ 1620
        L  D  P  N  N  P  I  Y  Y  I  S  K  Q  L  V  L  V  C  K  D
```

Figure 6-2 continued

```
     ATCTGTATAAAGTTACTAACGATGACGGTATTAACGTAACAAAGATTATTGGAGAATTAT
1621 ---------+---------+---------+---------+---------+---------+ 1680
       L  Y  K  V  T  N  D  D  G  I  N  V  T  K  I  I  G  E  L  *

     AATACTGAAAACAAACCTATATCAAATAAATGGATAAAATTACTAGAAATATCAGAGAAG
1681 ---------+---------+---------+---------+---------+---------+ 1740
                                  M  D  K  I  T  R  N  I  R  E  G
                                  H2L
     GAATACATATATTGTTACCATTTTATGAAAATCTTCCTGATATTAGCCTAAGTTTAGGAA
1741 ---------+---------+---------+---------+---------+---------+ 1800
       I  H  I  L  L  P  F  Y  E  N  L  P  D  I  S  L  S  L  G  K

     AAAGTCCATTACCTAGTTTGGAGTATGGAACCAATTACTTTCTACAATTATCAAGAGTAA
1801 ---------+---------+---------+---------+---------+---------+ 1860
       S  P  L  P  S  L  E  Y  G  T  N  Y  F  L  Q  L  S  R  V  N

     ATGACCTAAATAGATTACCTACGGATATGTTGAGTTTATTTACACATGATATAATGTTGC
1861 ---------+---------+---------+---------+---------+---------+ 1920
       D  L  N  R  L  P  T  D  M  L  S  L  F  T  H  D  I  M  L  P

     CTGAAACAGATATGGAAAAGGTATATGATATACTTAATATAAAATCTGTAAAATCATACG
1921 ---------+---------+---------+---------+---------+---------+ 1980
       E  T  D  M  E  K  V  Y  D  I  L  N  I  K  S  V  K  S  Y  G

     GTAAAAGTATTAAAGCCGATGCTGTTGTTGCAGATCTAAGTGCTAGGAACAGATTATTCA
1981 ---------+---------+---------+---------+---------+---------+ 2040
       K  S  I  K  A  D  A  V  V  A  D  L  S  A  R  N  R  L  F  K

     AAAAAGATAGAGAATTGATTAAATCTAATAATTATCTCACGGATAATAATCTATATATAA
2041 ---------+---------+---------+---------+---------+---------+ 2100
       K  D  R  E  L  I  K  S  N  N  Y  L  T  D  N  N  L  Y  I  S

     GTGATTATAAAATGTTGACATTTGAAGTATTTAGACCGCTTTTTGATCTATCGTCGGAAA
2101 ---------+---------+---------+---------+---------+---------+ 2160
       D  Y  K  M  L  T  F  E  V  F  R  P  L  F  D  L  S  S  E  K

     AATATTGCATAGTAAAGTTACCCACGTTATTTGGTAAATGTGTTATAGATACGATTAGAG
2161 ---------+---------+---------+---------+---------+---------+ 2220
       Y  C  I  V  K  L  P  T  L  F  G  K  C  V  I  D  T  I  R  V

     TATACTGTAGTCTTTTTAAATCAGTTAGATTATTCAAATGTGCTAGCGATAGTTGGTTAA
2221 ---------+---------+---------+---------+---------+---------+ 2280
       Y  C  S  L  F  K  S  V  R  L  F  K  C  A  S  D  S  W  L  K

     AAGACAGCGCTATCATGGTCGCGAGTGATATATATAAAAAAAATATAGATATTTTTATGT
2281 ---------+---------+---------+---------+---------+---------+ 2340
       D  S  A  I  M  V  A  S  D  I  Y  K  K  N  I  D  I  F  M  S

     CACATATTAGATCTGTTTTAAAATCGCAATATTGGAAAGATTCTAATAACGTTCAGTTTA
2341 ---------+---------+---------+---------+---------+---------+ 2400
       H  I  R  S  V  L  K  S  Q  Y  W  K  D  S  N  N  V  Q  F  S
```

<u>Figure 6-2 continued</u>

```
      GTATATTGAAAGAATCTGTTGATAAGGAATTTATTAACAAATTTTTGGAATTTTCTACAT
2401 ---------+---------+---------+---------+---------+---------+ 2460
       I  L  K  E  S  V  D  K  E  F  I  N  K  F  L  E  F  S  T  S

      CTGTATACGAATCGTTATATTATGTACATTCATTATTATATTCTAGTATGATATCTGATA
2461 ---------+---------+---------+---------+---------+---------+ 2520
       V  Y  E  S  L  Y  Y  V  H  S  L  L  Y  S  S  M  I  S  D  N

      ATAAAAGTATAGAAAACGAATATCAAAAAAAATTGACAAAACTCTTATTATAAGAAATCC
2521 ---------+---------+---------+---------+---------+---------+ 2580
       K  S  I  E  N  E  Y  Q  K  K  L  T  K  L  L  L  *

      GTACAGATAAATGAGTAGTTATCATGCAGCATATATTGATTACGCACTTAGAGTTACGGA
2581 ---------+---------+---------+---------+---------+---------+ 2640
                   M  S  S  Y  H  A  A  Y  I  D  Y  A  L  R  V  T  E
                   H3L
      ATCTATGACCGATACAATGGGAACAGATACAGAAATTACATTAAAACCTTATCAACATTT
2641 ---------+---------+---------+---------+---------+---------+ 2700
       S  M  T  D  T  M  G  T  D  T  E  I  T  L  K  P  Y  Q  H  F

      TGTAGCTCGTGTTTTTTTAGGATTAGATAAAATGCATTCATTATTACTATTTCATGACAC
2701 ---------+---------+---------+---------+---------+---------+ 2760
       V  A  R  V  F  L  G  L  D  K  M  H  S  L  L  L  F  H  D  T

      AGGTGTTGGTAAAACTATTACAACAACGTTTATTATTAAACAATTAAAAAATATATACAC
2761 ---------+---------+---------+---------+---------+---------+ 2820
       G  V  G  K  T  I  T  T  T  F  I  I  K  Q  L  K  N  I  Y  T

      AAATTGGTCTATATTATTGCTGGTTAAAAAGCACTTG
2821 ---------+---------+---------+------- 2857
       N  W  S  I  L  L  L  V  K  K  H  L
```

Figure 6-2 continued

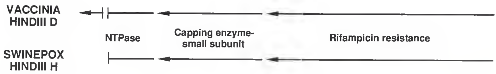
including the length of the peptides and the percent identity at the amino acid level, are shown in Figure 6-3.

The SPV open reading frame H1L encodes a peptide of 551 amino acids. Comparison of this orf to sequences within the genbank database revealed a high degree of homology to vaccinia orf D13L. This orf represents the locus to which resistance of vaccinia virus to the antibiotic rifampicin has been mapped (Seto et al, 1987; Tartaglia and Paoletti, 1985; Baldick and Moss, 1987). The vaccinia orf is also 551 amino acids in length and there is 70.4% identity at the amino acid level to SPV H1L.

The SPV open reading frame H2L encodes a polypeptide of 287 amino acids, the exact length of its counterpart in vaccinia, orf D12L. For vaccinia, this open reading frame encodes the small subunit of the messenger RNA capping enzyme (Niles et al., 1989). The identity at the amino acid level between SPV orf H2L and the vaccinia enzyme is 77.0%.

Downstream and adjacent to the capping enzyme in the vaccinia genome is the gene encoding a nucleotide triphosphatase (Rodriguez et al., 1986; Broyles and Moss, 1987; Seto et al., 1987). The open reading frame for this enzyme encodes a 631 amino acid polypeptide. The sequence for SPV downstream of H2L (designated orf H3L) consists of 267 nuleotides encoding a polypeptide of at least 89 amino acids, before the sequenced region ends. However, a comparison of these 89 amino acids to the amino terminal 89

A



B

SPV ORF	GENE	PEPTIDE LENGTH	PEPTIDE HOMOLOGY
H1L	Rifampicin resistance	VV-551 SPV-551	70.4%
H2L	Capping enzyme	VV-287 SPV-287	77.0%
H3L	NTPase	VV-631 SPV-89+	69.7%

Figure 6-3. Swinepox virus core open reading frames comparison.
A. A representation of the colinearity of swinepox DNA conserved region sequence compared to the analogous region of vaccinia virus. The 3 open reading frames deduced from the sequence within the SPV HindIII H fragment are aligned with the analogous vaccinia open reading frames. The gene designations refer to data from vaccinia.
B. A comparison of the length of the open reading frames and the identity at the amino acid level between the SPV and vaccinia products are shown.

amino acids of the vaccinia NTPase reveals 69.7% identity. From the high degree of conservation seen in the 2 complete SPV orfs (H1L and H2L), it seems probable that SPV orf H3L represents the NTPase equivalent and probably has a total length similar to that of its vaccinia homolog.

An examination of the intergenic regions of the SPV sequence demonstrated that many of the characteristics present for vaccinia open reading frames are conserved. In vaccinia, the capping enzyme is expressed at both early andlate times, whereas the rifampicin resistance and NTPase genes are expressed only at late times. The region upstream of the putative translation initiation codon for all three SPV genes is A+T rich as are all vaccinia promotor regions. Approximately 20 bp upstream of the start site of the vaccinia capping enzyme gene is the sequence AATAATGAAAAC, differing from the vaccinia early promotor consensus motif, AAAAATGAAAA/TA, by only two nucleotides. The SPV capping enzyme has a similarly located sequence, AATACTGAAAAC, differing from the consensus by 3 nucleotides, and from the vaccinia sequence by only a single base (C at position 5). Located 27 nucleotides downstream of the translation termination codon of the SPV capping enzyme is the consensus early transcription termination signal TTTTTNT. This signal is also seen downstream of the vaccinia capping enzyme. In addition, the SPV homologues of both the capping enzyme and the NTPase genes contain the consensus late transcription

signal TAAATG, with the ATG being the translation initiation codon. The putative SPV rifampicin resistance gene, on the other hand, is preceeded by the sequence ACAATG, with the ATG again being the translation initiation site. Interestingly, the vaccinia rifampicin resistance gene also lacks the consensus late transcription signal, having the sequence CAAATG. For vaccinia, both the capping enzyme and the NTPase genes, similar to SPV, retain the normal TAAATG consensus.

Analysis of the SPV Terminal Region Sequence

Despite the conservation noted between the 2.85 kb of DNA from the central core of SPV to the analogous region of vaccinia virus, these two poxviruses are drastically different both in vitro and in natural and experimental infections in vivo. Therefore, sequence analysis of a region near the left terminus of SPV was undertaken with the hypothesis that this region should be quite different and thereby provide a genetic explanation for the phenoytpic dissimilarity between SPV and vaccinia. This analysis of the swinepox virus terminal region involved the sequencing of 7664 bp encompassing the region between the leftmost HindIII restriction site and the second BamHI site approximately 9.8 kb internal to the left terminal hairpin of the genome. The total A+T content of this sequence was found to be 72.3%. From this sequence, 10 complete open reading frames and 1

partial reading frame were deduced. The orientation and location relative to the SPV genome of these orf's are shown in Figure 6-4. The nucleotide sequence, and deduced open reading frames, designated C1L through C11L, are shown in Figure 6-5. Each open reading frame is transcribed in the leftward orientation, towards the terminus of the DNA, as is also seen with the majority of the open reading frames in the analogous region of the vaccinia genome. However, the high degree of homology noted between the SPV and vaccinia orfs within the conserved central core is remarkably absent in the terminal region. We have characterized each open reading frame in terms of size, molecular weight of the predicted polypeptide and homology to other viral and cellular proteins. We have examined the orf's for signal leader sequences, which are required for the insertion of secreted and membrane bound proteins into the endoplasmic reticulum, and consensus modification signals such as those for N-linked glycosylation and phosphorylation.

The open reading frame C1L, nearest the terminus, potentially encodes a polypeptide of 340 amino acids with a molecular weight of 39.6 kD. No significant homologs were found in database searches. The protein has a possible leader signal no additional transmembrane domains, and 4 potential glycosylation and 14 phosphorylation sites. Due to these features we would predict that the C1L orf would encode a protein that would either be secreted or membrane

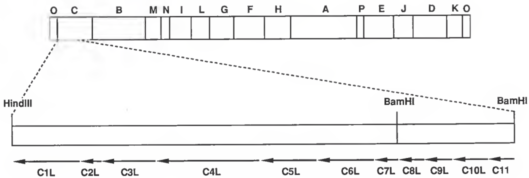


Figure 6-4. Swinepox virus terminal open reading frames. Schematic representation of the location and orientation of open reading frames deduced from the sequence near the left terminus of swinepox virus DNA. The genomic HindIII map for SPV is shown with the 6.6 kb region that was sequenced expanded. Each arrow represents an individual open reading frame with the designation given each open reading frame shown below.

Figure 6-5. Complete nucleotide sequence and the deduced amino acid sequences near the left terminus of swinepox virus. Nucleotide number 1 begins at the BamHI site approximately 9.7 kb from the left terminus and proceeds towards the hairpin. The deduced amino acids are indicated below the first nucleotide of each codon triplet, and the open reading frame designation below the initiating methionine.

```
     GGATCCGCTGGATATGATCTGTATAGTGCATATAGTTATACAGTTAAGCCGTATAATAGA
   1 ---------+---------+---------+---------+---------+---------+ 60
     G  S  A  G  Y  D  L  Y  S  A  Y  S  Y  T  V  K  P  Y  N  R
     C11L
     ATTTAGTTAGAACAGATATTTGTTTAATGATACCAGATAAATGTTATGGACGCATATCG
  61 ---------+---------+---------+---------+---------+---------+ 120
     I  L  V  R  T  D  I  C  L  M  I  P  D  K  C  Y  G  R  I  S

     CCTAGATCGGGATTATCGTTAAATTATAATATAGATATAGGAGGAGGCGTTATTGATAGT
 121 ---------+---------+---------+---------+---------+---------+ 180
     P  R  S  G  L  S  L  N  Y  N  I  D  I  G  G  G  V  I  D  S

     GATTACAGAGGGGAAATAGGTATCGTGTTTATAAATAATGGATGTAGTGATTTTAACATA
 181 ---------+---------+---------+---------+---------+---------+ 240
     D  Y  R  G  E  I  G  I  V  F  I  N  N  G  C  S  D  F  N  I

     AAGGTAGGTGATAGGATAGCACAAATAATATTTGAAAGAGTAGAATATCCTATAATGGAA
 241 ---------+---------+---------+---------+---------+---------+ 300
     K  V  G  D  R  I  A  Q  I  I  F  E  R  V  E  Y  P  I  M  E

     GAAGTAAAATGTTTGGAAGATACAGAACGTGGAAATAGTGGTTTTGGGTCAAGTGGTATG
 301 ---------+---------+---------+---------+---------+---------+ 360
     E  V  K  C  L  E  D  T  E  R  G  N  S  G  F  G  S  S  G  M

     TAAAGTATAATAAATGAAAAAATAATTCATCTGTATTATATCCATTATTATCAATATGTA
 361 ---------+---------+---------+---------+---------+---------+ 420
     *                                                      M  Y
                                                            C10L
     CAAGAAATATAACTCTAACGTATGCATTAGGAATGTATTATATGTATATCTAAAATATAA
 421 ---------+---------+---------+---------+---------+---------+ 480
      K  K  Y  N  S  N  V  C  I  R  N  V  L  Y  V  Y  L  K  Y  N

     TACTATAAATAAACTTAGTAGATATGAACGGATGATATACACAAAGATAAAAAATCAATG
 481 ---------+---------+---------+---------+---------+---------+ 540
      T  I  N  K  L  S  R  Y  E  R  M  I  Y  T  K  I  K  N  Q  C

     TGAAGCGATAAAATACAGATATTGTAATGATTTTAATTCTGTTACATGTATTTTAGAATA
 541 ---------+---------+---------+---------+---------+---------+ 600
      E  A  I  K  Y  R  Y  C  N  D  F  N  S  V  T  C  I  L  E  Y

     CGATGAAAATAAGTATATAGATAACGTGCATAAAGAAGTTATTAGTATATTGTTATCAGA
 601 ---------+---------+---------+---------+---------+---------+ 660
      D  E  N  K  Y  I  D  N  V  H  K  E  V  I  S  I  L  L  S  D

     TTCGCGACCTAGTATCAAATTAGCTGCTATTTCGTTATTATCTATAATAATAGATAAACT
 661 ---------+---------+---------+---------+---------+---------+ 720
      S  R  P  S  I  K  L  A  A  I  S  L  L  S  I  I  I  D  K  L

     AATATGTAGAAATATTCGTATAGCTAAATATATAATTGATGATATAATAAATATTATATC
 721 ---------+---------+---------+---------+---------+---------+ 780
      I  C  R  N  I  R  I  A  K  Y  I  I  D  D  I  I  N  I  I  S

     AGAAGACGGTATATATATTATATTATTTTTAGATGAATTTGATAAATATACCGATACCCG
 781 ---------+---------+---------+---------+---------+---------+ 840
      E  D  G  I  Y  I  I  L  F  L  D  E  F  D  K  Y  T  D  T  R
```

```
      ATGTAGGCGCCGTGGATTAAGTATGATGATAGCGAGCATTGTAACTTACTACTGTTTACG
  841 ---------+---------+---------+---------+---------+---------+ 900
       C  R  R  R  G  L  S  M  M  I  A  S  I  V  T  Y  Y  C  L  R

      GTATGTATTAAAAATATAAAAATAAATCTTTTTTTTAAAAATGAACCGTAATATGTGGA
  901 ---------+---------+---------+---------+---------+---------+ 960
       Y  V  L  K  I  *                        M  N  R  N  M  W  I
                                               C9L
      TAGTGTTATCGTGTGTATTATATATGATTTATATATGTAACGGACGAGATGTATTGTTAT
  961 ---------+---------+---------+---------+---------+---------+ 1020
        V  L  S  C  V  L  Y  M  I  Y  I  C  N  G  R  D  V  L  L  Y

      ATCCACCACATAAGAAAACAAATAAGGTTATAGTAAAATGTAACGGATATACTAATTCTA
 1021 ---------+---------+---------+---------+---------+---------+ 1080
        P  P  H  K  K  T  N  K  V  I  V  K  C  N  G  Y  T  N  S  T

      CGTATAGTATCTTATATTGGATGGTAGGTAACAACAATACATTCGTAGAACAACTAAATA
 1081 ---------+---------+---------+---------+---------+---------+ 1140
        Y  S  I  L  Y  W  M  V  G  N  N  N  T  F  V  E  Q  L  N  S

      GCGATCATTATAAAGAGAAGAAATACAATAGTACTGAAAAAAATGAGCATATGTATAAGT
 1141 ---------+---------+---------+---------+---------+---------+ 1200
        D  H  Y  K  E  K  K  Y  N  S  T  E  K  N  E  H  M  Y  K  L

      TACGTACCGATCTTATTATATATAATATTACGTCAGAAATGGAGATGACAAAACTAACAT
 1201 ---------+---------+---------+---------+---------+---------+ 1260
        R  T  D  L  I  I  Y  N  I  T  S  E  M  E  M  T  K  L  T  C

      GTGTATTATCAGATATATACACACCTATCAAGGCATCTATAATATTAAATAATTTATGGA
 1261 ---------+---------+---------+---------+---------+---------+ 1320
        V  L  S  D  I  Y  T  P  I  K  A  S  I  I  L  N  N  L  W  S

      GTTGTTTAAATACTACACAAGTATGAAATATGAAATATAAAGTATTCAAATAAATAACAA
 1321 ---------+---------+---------+---------+---------+---------+ 1380
        C  L  N  T  T  Q  V  *

      TAATGTCAACTATGAATACGTTGGCATTTTGTTATGGATTACCTAACATAAATGATATCA
 1381 ---------+---------+---------+---------+---------+---------+ 1440
        M  S  T  M  N  T  L  A  F  C  Y  G  L  P  N  I  N  D  I  T
        C8L
      CGCAAGGTATAATTTTTGTTAGAAATAACATATTTTACTCATATTTAACAGATTATGCAA
 1441 ---------+---------+---------+---------+---------+---------+ 1500
        Q  G  I  I  F  V  R  N  N  I  F  Y  S  Y  L  T  D  Y  A  M

      TGGAAGCGTGTATATTGAATTATATAAATATTAGAGCCGATAAAATAGAAGATCTAAAGA
 1501 ---------+---------+---------+---------+---------+---------+ 1560
        E  A  C  I  L  N  Y  I  N  I  R  A  D  K  I  E  D  L  K  K

      AATCATTAGTTGGAAAAACTATTAGCGTGAGAGTTATTAGAGTTGATGTATTAAAAGGAT
 1561 ---------+---------+---------+---------+---------+---------+ 1620
        S  L  V  G  K  T  I  S  V  R  V  I  R  V  D  V  L  K  G  Y
```

<u>Figure 6-5 continued</u>

```
     ATATAGATGTTTCAATTGTATAATTTTTTTATCAAAACTGAAGTATAATCTAGACCTTAG
1621 ---------+---------+---------+---------+---------+---------+ 1680
      I  D  V  S  I  V  *

     AAGATATTTTGTACCATATAAAATGGATCCTGTTTGTTGGATATGTAAAGATGACTACAG
1681 ---------+---------+---------+---------+---------+---------+ 1740
                           M  D  P  V  C  W  I  C  K  D  D  Y  S
                           C7L
     TATTGAAAAGAATTATTGTAACTGTAAAAACGAGTATAAAGTTGTACACGATGAATGTAT
1741 ---------+---------+---------+---------+---------+---------+ 1800
      I  E  K  N  Y  C  N  C  K  N  E  Y  K  V  V  H  D  E  C  M

     GAAAAAGTGGATACAATACTCAAGGGAACGATCTTGTAAATTATGTAATAAAGAATATAA
1801 ---------+---------+---------+---------+---------+---------+ 1860
      K  K  W  I  Q  Y  S  R  E  R  S  C  K  L  C  N  K  E  Y  N

     CATCATTAGTGTTAGAAAACCATTCTCACAGTGGGTATTCTCCATTAAAGATTGCAAAAA
1861 ---------+---------+---------+---------+---------+---------+ 1920
      I  I  S  V  R  K  P  F  S  Q  W  V  F  S  I  K  D  C  K  K

     GTCAGCAATTTTGTACGCTACTCTATTCTTATGTACGTTTATTATATCGCTTGTTTTAAC
1921 ---------+---------+---------+---------+---------+---------+ 1980
      S  A  I  L  Y  A  T  L  F  L  C  T  F  I  I  S  L  V  L  T

     TAGAATTAATATAACAAAAATAATAGATACATCAAAAAATGATGTTTCATTTAAGCTGGT
1981 ---------+---------+---------+---------+---------+---------+ 2040
      R  I  N  I  T  K  I  I  D  T  S  K  N  D  V  S  F  K  L  V

     TACGATGATATTCTACTTATTACCATTTGTCATAACTTGTATATCGTTCATAACGCTGAT
2041 ---------+---------+---------+---------+---------+---------+ 2100
      T  M  I  F  Y  L  L  P  F  V  I  T  C  I  S  F  I  T  L  I

     AGTTTATCTATATAAATATTGTAAGATTTCCGCTAAAAACAACACATACGATACGATTTA
2101 ---------+---------+---------+---------+---------+---------+ 2160
      V  Y  L  Y  K  Y  C  K  I  S  A  K  N  N  T  Y  D  T  I  Y

     TGAACTTTAAAGTGAAAATTTAATCTATTTTTATAATAAAACATGCATTTCATATTCATT
2161 ---------+---------+---------+---------+---------+---------+ 2220
      E  L  *                                   M  H  F  I  F  I
                                                C6L
     ATATTATCACTATCATTTGTAGTAAATGCCGATGTATTTCCATCGTCGGTTACTTTATCA
2221 ---------+---------+---------+---------+---------+---------+ 2280
      I  L  S  L  S  F  V  V  N  A  D  V  F  P  S  S  V  T  L  S

     TCTAATGATTTTGATACAATAATTAAATGGGATAATAATGTAATATCATACGATGTAGAA
2281 ---------+---------+---------+---------+---------+---------+ 2340
      S  N  D  F  D  T  I  I  K  W  D  N  N  V  I  S  Y  D  V  E

     TTAATGCAGTACAGTCATGACGAATGGAGAACCGTTTGTACTAATTCTTTAGGATACTGT
2341 ---------+---------+---------+---------+---------+---------+ 2400
      L  M  Q  Y  S  H  D  E  W  R  T  V  C  T  N  S  L  G  Y  C
```

<u>Figure 6-5 continued</u>

```
     AATTTAACAAATTCTGATATCGACAATGATGATGAAACATGGGTGAGGTTTAAATATGAA
2401 ---------+---------+---------+---------+---------+---------+ 2460
     N  L  T  N  S  D  I  D  N  D  D  E  T  W  V  R  F  K  Y  E

     AATAAGACATCTAATGAACATAATATTGGCAGAGTATGTGAGATTGTACAAATAACTTCA
2461 ---------+---------+---------+---------+---------+---------+ 2520
     N  K  T  S  N  E  H  N  I  G  R  V  C  E  I  V  Q  I  T  S

     CCTATTGTTAACATGACAAGAGATGGTTCAATTATACTATTAGATATACATCATCCAATG
2521 ---------+---------+---------+---------+---------+---------+ 2580
     P  I  V  N  M  T  R  D  G  S  I  I  L  L  D  I  H  H  P  M

     ACATACGATAATCAGTATTATATATATAATAATATAACATTATGTGGATTTGAATTTATT
2581 ---------+---------+---------+---------+---------+---------+ 2640
     T  Y  D  N  Q  Y  Y  I  Y  N  N  I  T  L  C  G  F  E  F  I

     TACGAAGCTACATTTATTATTAATGATACAATTATACCATATAGTATAGACAATCAATAT
2641 ---------+---------+---------+---------+---------+---------+ 2700
     Y  E  A  T  F  I  I  N  D  T  I  I  P  Y  S  I  D  N  Q  Y

     TGTGATGATGTTCATTGTTTATTTTACTTTATATCACAAGAACCCGTTTGTGTGTATGTA
2701 ---------+---------+---------+---------+---------+---------+ 2760
     C  D  D  V  H  C  L  F  Y  F  I  S  Q  E  P  V  C  V  Y  V

     ATGGGTATGGAACAATATTATGAATTTGGTCCAAAAAAAACAGATAATAGTACTAGAGTG
2761 ---------+---------+---------+---------+---------+---------+ 2820
     M  G  M  E  Q  Y  Y  E  F  G  P  K  K  T  D  N  S  T  R  V

     TGTGTAGATGGATTAATTCCAAGAAAAATCGATACATATTTTATTAAAGATTTCGATGAT
2821 ---------+---------+---------+---------+---------+---------+ 2880
     C  V  D  G  L  I  P  R  K  I  D  T  Y  F  I  K  D  F  D  D

     ATAGATAGAGTTAATAACAGATTATATAGAGTTGTAAGTGATAAATATGAATCCAATATA
2881 ---------+---------+---------+---------+---------+---------+ 2940
     I  D  R  V  N  N  R  L  Y  R  V  V  S  D  K  Y  E  S  N  I

     TCGTCAAAGTTTATGCACTTATATAATAATATATTATCTTCGTTTAAACTAATATTGCAA
2941 ---------+---------+---------+---------+---------+---------+ 3000
     S  S  K  F  M  H  L  Y  N  N  I  L  S  S  F  K  L  I  L  Q

     GAACTTATGGTAAATACTGAACAGTAAATACGTTTATAAAGATAAAGGAATGAATTCGTA
3001 ---------+---------+---------+---------+---------+---------+ 3060
     E  L  M  V  N  T  E  Q  *                       M  N  S  Y
                                                     C5L
     TATTGTAATAAAAAATTCATTACGTGATTATAGATCTGGAAGAATTATAAGAAAATACAT
3061 ---------+---------+---------+---------+---------+---------+ 3120
      I  V  I  K  N  S  L  R  D  Y  R  S  G  R  I  I  R  K  Y  I

     AAGAAAATTAAATAAGGATGAGTATAAGCATTTTTGTGCTGTATTTAGATTAAATGTAGA
3121 ---------+---------+---------+---------+---------+---------+ 3180
      R  K  L  N  K  D  E  Y  K  H  F  C  A  V  F  R  L  N  V  D
```

Figure 6-5 continued

```
      TTTTTCTCAAGATGATAAAAATCCATCTAGAAAAGAAGTAATAAGAATAATAGATGAGGA
3181 ---------+---------+---------+---------+---------+---------+ 3240
       F  S  Q  D  D  K  N  P  S  R  K  E  V  I  R  I  I  D  E  E

      ATTCAATTTTGTGATCTTAGACTATTTTATGATATCATGACCGTTGTACCTAATCATAT
3241 ---------+---------+---------+---------+---------+---------+ 3300
       F  N  F  C  D  L  R  L  F  Y  D  I  M  T  V  V  P  N  H  M

      GAATGTGGCATCTATTATTTATAGCGAATACGAATATCTTTTAAAAAAATCAAATTATAA
3301 ---------+---------+---------+---------+---------+---------+ 3360
       N  V  A  S  I  I  Y  S  E  Y  E  Y  L  L  K  K  S  N  Y  K

      AAATAAGAAGATAAATTATACTATATTAGATAAGATTAATAAATATCATAGTATAGATGA
3361 ---------+---------+---------+---------+---------+---------+ 3420
       N  K  K  I  N  Y  T  I  L  D  K  I  N  K  Y  H  S  I  D  D

      TATTATATTTATGTATCTTCATTGGAGAAAAAAATATAACAACACATGCGCATGTGGTAA
3421 ---------+---------+---------+---------+---------+---------+ 3480
       I  I  F  M  Y  L  H  W  R  K  K  Y  N  N  T  C  A  C  G  K

      GTTATTTAAGGAACTCATGAAATATGATATATTAGCTACAAAATATATATATAATGATAT
3481 ---------+---------+---------+---------+---------+---------+ 3540
       L  F  K  E  L  M  K  Y  D  I  L  A  T  K  Y  I  Y  N  D  I

      TATAAATACATACAAAGAGGGAGATACTATATCCATTAACATACGTTTAAAATGTAAAGA
3541 ---------+---------+---------+---------+---------+---------+ 3600
       I  N  T  Y  K  E  G  D  T  I  S  I  N  I  R  L  K  C  K  D

      TGATATAATTAAACATTGTAAGTCTTCTATAGGTATGTTTGCTATATTATCATCGAAAAT
3601 ---------+---------+---------+---------+---------+---------+ 3660
       D  I  I  K  H  C  K  S  S  I  G  M  F  A  I  L  S  S  K  I

      AATCGACGTAGATTTTGATGTTATATTCTTTTCACAAATAAGTATAAGATATAGACTAAT
3661 ---------+---------+---------+---------+---------+---------+ 3720
       I  D  V  D  F  D  V  I  F  F  S  Q  I  S  I  R  Y  R  L  I

      ATTCAAAAAATATCTCATACAATCATTATACTTACAATAATAATTGTTTTTTTTTTGAAA
3721 ---------+---------+---------+---------+---------+---------+ 3780
       F  K  K  Y  L  I  Q  S  L  Y  L  Q  *

      AATAATCCTAAATCTATCATAACATGAATTCATTATTATTACGATTACATGATTTTTTTA
3781 ---------+---------+---------+---------+---------+---------+ 3840
                               M  N  S  L  L  L  R  L  H  D  F  F  K
                               C4L
      AACATGGAATTATGTGTGATATAAAAATAGTATCCATAGAGAATAATAAAACCATTAGCG
3841 ---------+---------+---------+---------+---------+---------+ 3900
        H  G  I  M  C  D  I  K  I  V  S  I  E  N  N  K  T  I  S  A

      CACATAGGTTAATATTATCTATGTACTCTAAGTACTTTTATAATATATTTAATTCAGATT
3901 ---------+---------+---------+---------+---------+---------+ 3960
        H  R  L  I  L  S  M  Y  S  K  Y  F  Y  N  I  F  N  S  D  F
```

Figure 6-5 continued

```
     TTATTGATAAAAATAATGATGAAATCTATATATGCGCCGATTATGATATATTGTATATTA
3961 ---------+---------+---------+---------+---------+---------+ 4020
       I  D  K  N  N  D  E  I  Y  I  C  A  D  Y  D  I  L  Y  I  I

     TATTGGAATTTATGTACACCGGTAATATAGTACTAACAAAGGATAATATAGAATTAGTAA
4021 ---------+---------+---------+---------+---------+---------+ 4080
       L  E  F  M  Y  T  G  N  I  V  L  T  K  D  N  I  E  L  V  I

     TACAAGTCTGTGATTATCTATGTATAGATTCTTTAATAAAAATATGTGAAGAATATATAT
4081 ---------+---------+---------+---------+---------+---------+ 4140
       Q  V  C  D  Y  L  C  I  D  S  L  I  K  I  C  E  E  Y  I  C

     GCGGTATAATAGATGAAACAAATTGTATACATCTCTTAAACTTTTCAGATACTTACAATC
4141 ---------+---------+---------+---------+---------+---------+ 4200
       G  I  I  D  E  T  N  C  I  H  L  L  N  F  S  D  T  Y  N  L

     TACAACGATTACGTGAAATGTCAAAATGGTATTTACCAAAAATAATAAATAATAACAAAC
4201 ---------+---------+---------+---------+---------+---------+ 4260
       Q  R  L  R  E  M  S  K  W  Y  L  P  K  I  I  N  N  N  K  L

     TGGTAGTAGAATTAGATATAGATGATATGATATTAATTATAAAAGAAATTAAATACATTG
4261 ---------+---------+---------+---------+---------+---------+ 4320
       V  V  E  L  D  I  D  D  M  I  L  I  I  K  E  I  K  Y  I  A

     CATGTGAATATATAGTTAAAAAAATAATATTAAATTGGATCGTTCATAAAGATGAACGAA
4321 ---------+---------+---------+---------+---------+---------+ 4380
       C  E  Y  I  V  K  K  I  I  L  N  W  I  V  H  K  D  E  R  I

     TTATTTATACTAAAAAATTAATGAAACATATCAATGATCAAGACCATTATACATCCTTAT
4381 ---------+---------+---------+---------+---------+---------+ 4440
       I  Y  T  K  K  L  M  K  H  I  N  D  Q  D  H  Y  T  S  L  S

     CGGATATTGAATTGTACAATAATATACGGGAACGAATATATGATAACAAAGAACACGATG
4441 ---------+---------+---------+---------+---------+---------+ 4500
       D  I  E  L  Y  N  N  I  R  E  R  I  Y  D  N  K  E  H  D  V

     TAGATATATCACATAACTTTATAATAATGGTAGGAGGAAAAAAGATATTTAATATAACCG
4501 ---------+---------+---------+---------+---------+---------+ 4560
       D  I  S  H  N  F  I  I  M  V  G  G  K  K  I  F  N  I  T  A

     CATTCAATCCGTTATCGAATAAAAAACATATTATAGACAGATACGATGATATGTTTGGTT
4561 ---------+---------+---------+---------+---------+---------+ 4620
       F  N  P  L  S  N  K  K  H  I  I  D  R  Y  D  D  M  F  G  C

     GTAAAACTCATTTTAGTGTTGTATACTTAAATAGTATACTATATATTATCGGTGGAAAGA
4621 ---------+---------+---------+---------+---------+---------+ 4680
       K  T  H  F  S  V  V  Y  L  N  S  I  L  Y  I  I  G  G  K  K

     AACGAGGATATTTCACTAAAGAGGTGTTGTCATATAATATAAAAAACAAATTATGGTGTT
4681 ---------+---------+---------+---------+---------+---------+ 4740
       R  G  Y  F  T  K  E  V  L  S  Y  N  I  K  N  K  L  W  C  Y
```

Figure 6-5 continued

```
     ACGAACCAGAATTAAATTATTTTAGATACGATACATCTGTATGTGTATCAAATGGGATGA
4741 ---------+---------+---------+---------+---------+---------+ 4800
       E  P  E  L  N  Y  F  R  Y  D  T  S  V  C  V  S  N  G  M  I

     TATATTCAATTGGTGGAAAAGATACAAATGGATATATGACAAACATCGTAGAATTTTGGA
4801 ---------+---------+---------+---------+---------+---------+ 4860
       Y  S  I  G  G  K  D  T  N  G  Y  M  T  N  I  V  E  F  W  K

     AACCTGAATGGAAATCATGGTATGATGGTCAACATTTGTGTTATCCTAGATGTTATATGT
4861 ---------+---------+---------+---------+---------+---------+ 4920
       P  E  W  K  S  W  Y  D  G  Q  H  L  C  Y  P  R  C  Y  M  S

     CGTTGGTAGACTATAATAATGAAGTATATACAATAGGTGGATTAAAAACATCAATAACGG
4921 ---------+---------+---------+---------+---------+---------+ 4980
       L  V  D  Y  N  N  E  V  Y  T  I  G  G  L  K  T  S  I  T  D

     ATGAATTTAATATAGAAATGATTGTATCAGACGATGCCGTAGAGAAACTGACCGATCATT
4981 ---------+---------+---------+---------+---------+---------+ 5040
       E  F  N  I  E  M  I  V  S  D  D  A  V  E  K  L  T  D  H  S

     CATGGATGAAGTTAAAACAATTTCCCATAGCAAAGAGTGGTATATCATCCATAGTATATA
5041 ---------+---------+---------+---------+---------+---------+ 5100
       W  M  K  L  K  Q  F  P  I  A  K  S  G  I  S  S  I  V  Y  N

     ACGATTTTATATACTGTATAGGTGGTCGTATAGATACACCACATATAAGTATAGAACACA
5101 ---------+---------+---------+---------+---------+---------+ 5160
       D  F  I  Y  C  I  G  G  R  I  D  T  P  H  I  S  I  E  H  T

     CTAACGATGTTTATATATATTCTTCAAGAGATGATTGTTGGAAATATTTATCAAATACAA
5161 ---------+---------+---------+---------+---------+---------+ 5220
       N  D  V  Y  I  Y  S  S  R  D  D  C  W  K  Y  L  S  N  T  N

     ATGTAAAAAGATCATTTTGTCTATCGTGTGTTTTTAATAATGAATTATATATAATAGGTG
5221 ---------+---------+---------+---------+---------+---------+ 5280
       V  K  R  S  F  C  L  S  C  V  F  N  N  E  L  Y  I  I  G  G

     GATATAATACAAACAGTGTAGAAAAGTACAATAAATTAAAAAATACATGGAAGCGTTTAA
5281 ---------+---------+---------+---------+---------+---------+ 5340
       Y  N  T  N  S  V  E  K  Y  N  K  L  K  N  T  W  K  R  L  N

     ACGATATTCCTAAGTTTGAAGAATGTGTTAATGAAGCATCGGCAATATATTTGTAGTATC
5341 ---------+---------+---------+---------+---------+---------+ 5400
       D  I  P  K  F  E  E  C  V  N  E  A  S  A  I  Y  L  *

     CCTTATAGCGTTCAAAAGAAACAAATCCATAACAGACATCTATATTCTTAATCTATCTAT
5401 ---------+---------+---------+---------+---------+---------+ 5460
                                                             M
                                                             C3L
     GTCAGATTGTATATTCGTATTTCAGATTCCGTTCATTGTGTATAGTAAACTCGATCAATG
5461 ---------+---------+---------+---------+---------+---------+ 5520
      S  D  C  I  F  V  F  Q  I  P  F  I  V  Y  S  K  L  D  Q  W
```

Figure 6-5 continued

```
     GATTTTTGGGAATATACTATGTAAAATAATGTCCGTATTATACTACGTAGGATTCTTTAG
5521 ---------+---------+---------+---------+---------+---------+ 5580
      I  F  G  N  I  L  C  K  I  M  S  V  L  Y  Y  V  G  F  F  S

     TAATATGTTTATAATAACACTTATGAGTATAGATAGATATTTTGCGATCGTTCATCCTAT
5581 ---------+---------+---------+---------+---------+---------+ 5640
      N  M  F  I  I  T  L  M  S  I  D  R  Y  F  A  I  V  H  P  I

     AAAGCGACAACCGTATAGGACGAAACGTATAGGTATCCTTATGTGCTGTTCCGCTTGGTT
5641 ---------+---------+---------+---------+---------+---------+ 5700
      K  R  Q  P  Y  R  T  K  R  I  G  I  L  M  C  C  S  A  W  L

     ATTATCCTTGATATTATCTAGTCCCGTATCTAAACTATACGAGAATATTCCTCATATGTC
5701 ---------+---------+---------+---------+---------+---------+ 5760
      L  S  L  I  L  S  S  P  V  S  K  L  Y  E  N  I  P  H  M  S

     TAAAGATATATACCAATGTACTCTGACGAACGAGAATGACTCCATAATCGCATTCATAAA
5761 ---------+---------+---------+---------+---------+---------+ 5820
      K  D  I  Y  Q  C  T  L  T  N  E  N  D  S  I  I  A  F  I  K

     AAGACTGATGCAAATAGAGATCACTATATTGGGATTCCTGATACCTATAATCATATTCGT
5821 ---------+---------+---------+---------+---------+---------+ 5880
      R  L  M  Q  I  E  I  T  I  L  G  F  L  I  P  I  I  I  F  V

     ATATTGCTATTATAGAATTTTTCTACAGTGGTTAGATTAAGAAATAGACGAAAGTATAA
5881 ---------+---------+---------+---------+---------+---------+ 5940
      Y  C  Y  Y  R  I  F  S  T  V  V  R  L  R  N  R  R  K  Y  K

     ATCTATAAAAATTGTATTAATGATTGTTGTATGTTCTCTAATATGTTGGATTCCGCTCTA
5941 ---------+---------+---------+---------+---------+---------+ 6000
      S  I  K  I  V  L  M  I  V  V  C  S  L  I  C  W  I  P  L  Y

     TATCGTTCTAATGATAGCGACGATTGTTAGCTTATATACATCTAATATATTTAGACATCT
6001 ---------+---------+---------+---------+---------+---------+ 6060
      I  V  L  M  I  A  T  I  V  S  L  Y  T  S  N  I  F  R  H  L

     GTGCCTCTATCTAAACCTGGCCTATGCGATCACCTTTTCGGAGACTATCTCGTTAGCGCG
6061 ---------+---------+---------+---------+---------+---------+ 6120
      C  L  Y  L  N  L  A  Y  A  I  T  F  S  E  T  I  S  L  A  R

     TTGTTGTATAAATCCAATAATATATACACTGATAGGTGAACATGTTCGATCTCGTATATC
6121 ---------+---------+---------+---------+---------+---------+ 6180
      C  C  I  N  P  I  I  Y  T  L  I  G  E  H  V  R  S  R  I  S

     TAGCATATGTTCGTGTATATATAGAGACAATAGGATTAGGAAAAAACTCTTTTCACGAAA
6181 ---------+---------+---------+---------+---------+---------+ 6240
      S  I  C  S  C  I  Y  R  D  N  R  I  R  K  K  L  F  S  R  K

     ATCTTCTAGCAGTAGCAATATTATTTAGTTGTTATTTTCTTACAAAACACAAGTTATAAA
6241 ---------+---------+---------+---------+---------+---------+ 6300
      S  S  S  S  S  N  I  I  *
```

<u>figure 6-5 continued</u>

```
     TAATCATTACGTAATCATGCTATCGTATATTATTAATCCTTTGCTAAGTATTGTATACTT
6301 ---------+---------+---------+---------+---------+---------+ 6360
                     M  L  S  Y  I  I  N  P  L  L  S  I  V  Y  F
                     C2L
     TATATTAGGAAATGTATCTAAGCTGCTTACATATATACTTATGAAAATAATGATTTTTTT
6361 ---------+---------+---------+---------+---------+---------+ 6420
      I  L  G  N  V  S  K  L  L  T  Y  I  L  M  K  I  M  I  F  L

     ACTTCGTGCGGTGAATCCATACTCTCTGATATCTAACAGAGGTTGGCTGTCGCTGGATAG
6421 ---------+---------+---------+---------+---------+---------+ 6480
      L  R  A  V  N  P  Y  S  L  I  S  N  R  G  W  L  S  L  D  S

     TATAAATCCCTTTAAAAAGGAAAAGCGTAGGGAGTCTTTTCTATCTAGTCTAAATCCGTT
6481 ---------+---------+---------+---------+---------+---------+ 6540
      I  N  P  F  K  K  E  K  R  R  E  S  F  L  S  S  L  N  P  F

     TAGAAAAGAGGAAACAAAGAAAAAAGAAGGTTTCTTTTCTGGTTGGTTCGGATAATCTCT
6541 ---------+---------+---------+---------+---------+---------+ 6600
      R  K  E  E  T  K  K  K  E  G  F  F  S  G  W  F  G  *

     TTTATAATTGAAATAATATTCCAAAAATAAATCATAATGATTACTAAAGCGATTGTGATA
6601 ---------+---------+---------+---------+---------+---------+ 6660
                                         M  I  T  K  A  I  V  I
                                         C1L
     TTGTCTATTATTACAGCATATGTAGATGCTTCCGCATTCTTAGTATACAATTATACATAT
6661 ---------+---------+---------+---------+---------+---------+ 6720
     L  S  I  I  T  A  Y  V  D  A  S  A  F  L  V  Y  N  Y  T  Y

     ACTTTACAAGATGATAATCATCGATATGACTTCGAAGTCACCGATTATTTTAATGATATA
6721 ---------+---------+---------+---------+---------+---------+ 6780
     T  L  Q  D  D  N  H  R  Y  D  F  E  V  T  D  Y  F  N  D  I

     CTAATAAAACGTTTAAAACTAAATAGCGAGACAGGAAGACCAGAATTAAGAAATGAACCA
6781 ---------+---------+---------+---------+---------+---------+ 6840
     L  I  K  R  L  K  L  N  S  E  T  G  R  P  E  L  R  N  E  P

     CCAACATGGTTTAATGAGACTAAGATTAGATATTATCCGAAAAATAATTATAATTTTATG
6841 ---------+---------+---------+---------+---------+---------+ 6900
     P  T  W  F  N  E  T  K  I  R  Y  Y  P  K  N  N  Y  N  F  M

     TTCTGGCTAAATAGAATGAGTGAAACGCTAGATGAGATAAATAAACTTCCAGAAACGAGT
6901 ---------+---------+---------+---------+---------+---------+ 6960
     F  W  L  N  R  M  S  E  T  L  D  E  I  N  K  L  P  E  T  S

     AATCCTTACAAGACTATGTCCTTGACAATTGGATGTACTGATCTAAGACAACTTCAAGTA
6961 ---------+---------+---------+---------+---------+---------+ 7020
     N  P  Y  K  T  M  S  L  T  I  G  C  T  D  L  R  Q  L  Q  V

     AATTTCGGTTATGTTACTGTAGGTGGTAATATATGGACACGATTCGACCCCAAGAATAAA
7021 ---------+---------+---------+---------+---------+---------+ 7080
     N  F  G  Y  V  T  V  G  G  N  I  W  T  R  F  D  P  K  N  K
```

Figure 6-5 continued

```
     CGCTTTAGTAAAGTTAGATCACGTACATTTCCAAAGGTAGGAATGTTAACTGTTAAATCA
7081 ---------+---------+---------+---------+---------+---------+ 7140
     R  F  S  K  V  R  S  R  T  F  P  K  V  G  M  L  T  V  K  S

     CAACACTGGGAACGTGTTATGGAACATCTTGGATCAATGGTAACATTAACATGTCCGTTT
7141 ---------+---------+---------+---------+---------+---------+ 7200
     Q  H  W  E  R  V  M  E  H  L  G  S  M  V  T  L  T  C  P  F

     ACAGCGGATGATTATTATAAAATTTCTAAGGGATATATAGATAAGCCAGTTAAGCCTACT
7201 ---------+---------+---------+---------+---------+---------+ 7260
     T  A  D  D  Y  Y  K  I  S  K  G  Y  I  D  K  P  V  K  P  T

     GTTACAGTTACAGGAATTGAAAGAGGAGATAATACTACATTGATATGCACATTTGATAAT
7261 ---------+---------+---------+---------+---------+---------+ 7320
     V  T  V  T  G  I  E  R  G  D  N  T  T  L  I  C  T  F  D  N

     CATTATCCGTCGTCGGTCGCTGTTAAATGGTATAACATCGAGGACTTTGCTCCGGACTAT
7321 ---------+---------+---------+---------+---------+---------+ 7380
     H  Y  P  S  S  V  A  V  K  W  Y  N  I  E  D  F  A  P  D  Y

     CGTTATGATCCGTACGTAAATGAATTGCTTCCTGATACGGACTATCTACCGGGTGAACCA
7381 ---------+---------+---------+---------+---------+---------+ 7440
     R  Y  D  P  Y  V  N  E  L  L  P  D  T  D  Y  L  P  G  E  P

     GGATATCCGACTATAACTAGGAGATTAGGTGATAAATATTTATTTACATCATCACCTAGG
7441 ---------+---------+---------+---------+---------+---------+ 7500
     G  Y  P  T  I  T  R  R  L  G  D  K  Y  L  F  T  S  S  P  R

     GTTATGGTACCAACTATCATGTCTAATAGAATAGCATGTGTTGGATTTCATAGTACGTTA
7501 ---------+---------+---------+---------+---------+---------+ 7560
     V  M  V  P  T  I  M  S  N  R  I  A  C  V  G  F  H  S  T  L

     GAACCAAGCATATATAGATGTGTAAACTGCTCGGGACCTGAGCCTGTTTTACAATACCAG
7561 ---------+---------+---------+---------+---------+---------+ 7620
     E  P  S  I  Y  R  C  V  N  C  S  G  P  E  P  V  L  Q  Y  Q

     GGAGATAGAAGGAATGACTTGGAGGATGAGGAGGATTAAAGCTT
7621 ---------+---------+---------+---------+---- 7664
     G  D  R  R  N  D  L  E  D  E  E  D  *
```

Figure 6-5 continued

bound, depending on whether or not the signal is cleaved. Consistent with C1L being either secreted or membrane associated, this peptide contains the tripeptide RGD which is a cell attachment sequence, found in extracellular glycoprotein fibronectin, that is crucial for its interaction with its cell surface receptor (Rouslahti and Pierschbacher, 1986). This tripeptide has been found and shown to have the same activity in other proteins including fibrinogen (Gartner and Bennett, 1985; Plow et al., 1985), discoidin I (Springer et al., 1984), Type I collagen (Denhar et al., 1987), complement fragment C3bi (Wright et al., 1987), and von Willebrand coagulation factor (Haverstick et al., 1985). Recently, RGD sequences have been found on the surface of several bacterial and viral pathogens where they reportedly function as adhesin molecules. RGD sequences found in the filamentous hemagglutinin of the bacterium Bordetella pertussis and in the VP1 protein of the foot-and-mouth disease virus have been shown to be important for adherance to host cell membranes (Fox et al., 1989: Relman et al., 1990). Attachment in each case is mediated by a group of related receptors termed integrins (Tamkun et al., 1986) found on numerous cell types including endothelial, epithelial and fibroblast (Hayman et al., 1985). It is possible that the RGD domain of the C1L protein of SPV may serve a similar cell binding function and mediate the attachment of swinepox virus to host cells to initiate an

infection. Consistent with this possibility are hydrophobicity plots of the C1L polypeptide which predict that the RGD domain is located within a hydrophilic region and therefore likely to be exposed at the surface of the polypeptide. However, it should be noted that the RGD tripeptide alone does not constitute the entire attachment site within the above mentioned cell adherent polypeptides, as residues on each side of the RGD sequence have been shown to be important for binding specificity. There are also numerous proteins that contain the RGD sequence, but lack cellular adhesion properties, possibly due an inaccessible location of the tripeptide within the secondary structure of the molecule.

The C2L orf potentially encodes a peptide of 92 amino acids with a molecular weight of 10.8 kD. This protein is basic and has a leader signal sequence, a single potential glycosylation site, and 3 possible phosphorylation sites. There is a putative transmembrane domain which could serve as an membrane anchor and I would therefore predict that C2L is also a membrane protein. Interestingly, C2L has a high degree of homology (48% AA identity) to a small 80 AA orf near the terminus of Shope fibroma virus DNA (Upton et al, 1987a), despite the fact that all of the other corresponding Shope orfs within the same area are missing from SPV. The maintenance of this small orf in two apparently quite

divergent viruses suggests a possible important function for the encoded peptide.

The C3L open reading frame potentially encodes a polypeptide of 269 amino acids with a molecular weight of 31.5 kD. It has a leader signal sequence, a single N-glycosylation site, 6 phosphorylation sites, and is extremely basic with a charge of +20 at pH 7.0. C3L exhibits approximately 25% identity at the amino acid level with members of the G protein coupled receptor family. This family of receptors, which includes rhodopsin and beta-adrenergic receptors, has many diverse members with their common feature being the presence of 7 transmembrane domains and the ability to interact with G proteins (Gilman, 1987; Stryer and Bourne, 1986). SPV C3L also is predicted to have 7 transmembrane domains, and when compared to the amino acids conserved between the the members of this receptor family, the identity is well over 50%. Therfore, C3L is also most likely a membrane protein, and may serve a receptor function and/or be involved in the cellular signal transduction pathway, as are the members of the G protein-coupled receptor family.

The C4L orf potentially encodes a polypeptide of 530 amino acids with a predicted molecular weight of 62.6 kD. It does not contain a hydrophobic leader signal, but does have 4 potential transmembrane domains. The C4L peptide is acidic with a charge of -10 at pH7.0 and has 3 N-glycosylation

sites and 13 possible phosphorylation sites. This orf has a significant degree of homology (approximately 35% AA identity) to a series of 3 orfs found in tandem within the inverted terminal repeats of the leporipoxviruses, Shope fibroma, myxoma, and malignant rabbit fibroma viruses (Upton and McFadden, 1986; Upton et al., 1990). The C4L orf also exhibits 22% identity to vaccinia orf C2L (Goebel et al., 1990; Kotwal and Moss, 1988b). The function of these products in the leporipoxviruses and in vaccinia is not known.

The SPV open reading frames C5L, C8L and C10L potentially encode polypeptides of 236 AA (28.6 kD), 86 AA (9.8 kD), and 167 AA (19.9 kD), respectively. Each peptide is basic and lacks endoplasmic reticulum targeting signal leader sequences. Open reading frame C10L had no significant homology to any database peptides. The C5L orf was 30% identical at the amino acid level to vaccinia K7R, although quite a bit larger than the 149 amino acid vaccinia peptide (Boursnell et al., 1988). Open reading frame C8L, which exhibited 44.3% identity at the amino acid level to vaccinia orf K3L (Boursnell et al., 1988), contains a putative leader sequence, but lacks an additional transmembrane domain, and is therefore possibly secreted from infected cells. No function has yet been attributed to either the K7R or K3L vaccinia open reading frames, although K3L, which is 88 amino acids in length, has a limited degree of homology, as

does the SPV analogue, to human and rat translation initiation factor 2-alpha (Goebel et al., 1990).

Open reading frame C6L potentially encodes a peptide of 274 amino acids with a molecular weight of 32.2 kD. It is very acidic with a charge of -20 at neutral pH. Similar to most of the SPV terminal peptides, C6L also has a putative hydrophobic leader sequence along with 2 additional potential transmembrane domains, and is therefore also likely to be membrane bound. It has 7 possible N-glycosylation sites and 9 phosphorylation sites. The C6L orf exhibits a significant degree of homology to the gamma interferon receptor; 25% identity at the amino acid level. The gamma interferon receptor is a novel receptor of 489 amino acids with no homology to any other family of receptors (Aguet et al.,1988). It is believed to be composed of 3 regions; an extracellular receptor of about 240 AA, a transmembrane sequence, and a large 190 AA cytoplasmic tail. The homology of SPV C6L is to the extracellular receptor and transmembrane regions, but not to the cytoplasmic tail. Therefore, C6L may be another SPV polypeptide either functioning as, or merely derived from, a normal cellular receptor.

The polypeptide potentially encoded by open reading frame C7L is 155 amino acids in length with a molecular weight of 18.4 kD. It lacks homology to any known proteins and does not have a signal leader sequence. However, C7L

does not have a hydrophobic leader but rather two internal stretches of hydrophobic residues which could also serve as transport signals (Spiess and Lodish, 1986; Wickner and Lodish, 1985). Therefore, it may also be a membrane protein.

The SPV open reading frame C9L potentially encodes a polypeptide 134 amino acids in length with a predicted molecular weight of 15.8 kD. It contains a hydrophobic signal leader sequence and has 5 potential glycosylation sites and 5 possible phosphorylation sites. Internal hydrophobic domains suggest this polypeptide may also be membrane bound. There were no significant viral or cellular protein homologues identified upon database searches.

The SPV open reading frame C11L is of interest despite the fact that it is only a partial sequence because it exhibits very good homology with the caboxyl terminus of vaccinia orf F2L. The function of the vaccinia F2L orf product is not known, however, it has a significant degree of homology to retroviral pseudoproteases (Roseman and Slabaugh, 1990; Slabaugh and Roseman, 1989) and to a bacterial dUTPase (McGeoch, 1990). The level of homology between SPV orf C11L and vaccinia F2L is 59% amino acid identity. This conservation between two seemingly divergent viruses such as SPV and vaccinia, and the identification of a similar product in orf virus (Mercer et al., 1989), a poxvirus of sheep, again suggests a possible essential function for this gene product.

A listing of the SPV open reading frames, the predicted lengths and molecular weights of the deduced polypeptides, and homology at the amino acid level to other poxvirus or cellular proteins are shown in Table 6-1. Table 6-1 also characterizes the swinepox terminal orfs with regard to the presence or absence of potential endoplasmic reticulum leader signals, and for those with leader signals, potential N-gycosylation sites are listed. Table 6-1 also includes a list of the protein phosphorylation sites and other notable features present in the predicted polypeptides. It should be noted that the items included in Table 6-1 indicate potential signals for each protein predicted solely from amino acid sequence and does not necessarily mean that these signals function during synthesis of the mature polypeptide.

Four of the SPV open reading frames (C4L, C5L, C8L, and C11L) had a significant degree of homology to polypeptides encoded by vaccinia virus. Although SPV orf C11L is only a partial sequence, all of the other SPV orf's and their corresponding vaccinia homologues are of similar sizes. However, each of the vaccinia genes is located at least 25 kb from the left terminus of the genome, whereas all of the SPV orf's are within 10 kb of the left hairpin. The location and orientation of the homologous open reading frames of SPV and vaccinia relative to their respective genomes are shown in Figure 6-6.

Table 6-1. The open reading frames deduced from the left terminus of swinepox virus DNA.

Open Reading Frame	Amino acid Length	Molecular Weight[a]	Hydrophobic Leader[b]	N-Glcosylation Sites[c]
C1L	340	39.6	Yes	4
C2l	92	10.8	Yes	1
C3L	269	31.5	Yes	1
C4L	530	62.6	No	3
C5L	236	28.5	No	-
C6L	274	32.2	Yes	7
C7L	155	18.4	No	-
C8L	86	9.8	No	0
C9L	134	15.8	Yes	5
C10L	167	19.9	No	-
C11L	120+	13.5+	?	-

[a] Expressed in kilodaltons.
[b] Defined as a series of 15 or more hydrophobic amino acid residues near the NH_2 terminus.
[c] Potential sites predicted by the MacPattern computer program (Fuchs, 1990). N-glycosylation sites shown only for peptides with hydrophobic leaders.
[d] Transmembrane domains predicted by the ALOM computer program (Klein et al, 1985). Acidic and basic refer to the charge of a given polypeptide at pH 8.0.
[e] Charge of polypeptide at pH 7.0. Charges >0 represent basic proteins while charges <0 would be acidic peptides.
[f] Homologies detected using the computer program TFASTA (Devereux et al, 1984) to search the GenBank database.

Phospho Sites[c]	Transmemb Domains[d]	Charge[e]	Homology[f]
14	1	-4	None; RGD domain
3	2	+8	Shope near term. ORF-48%
6	7	+20	G prot. coupled receptors-25%
13	4	-10	Shope C6L,C8L,C9L-35% Vaccinia C2L-22%
14	0	+14	Vaccinia K7R-30%
9	3	-20	Shope C7L-28% Interferon-δ receptor-25%
6	2	+8	None
1	0	+1	Vaccinia K3L-44.3%
5	2	+4	None
3	0	+8	None
-	0	-	Vaccinia F2L-59% Retroviral pseudoprotease-34%

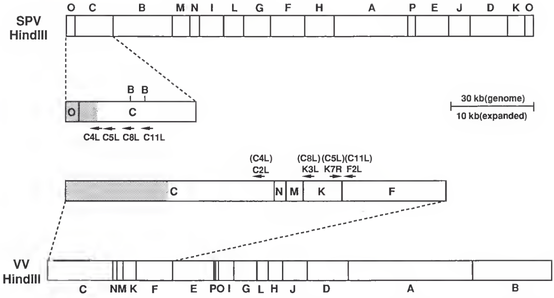


Figure 6-6. Swinepox virus and vaccinia virus open reading frame homologues. The <u>Hind</u>III maps for the SPV and vaccinia virus (VV) genomes are shown with the areas near the left terminus of each genome expanded. The 4 SPV orf's (C4L, C5L, C8L, and C11L) with homology to vaccinia orf's are shown relative to their locations within the expanded left end. Likewise, the 4 homologous vaccinia orf's (C2L, K3L, K7R, and F2L) are shown relative to the left end of the VV genome. The designation of the individual SPV homologues are also shown above the corresponding VV orf for which homology exists. The 30 kb scale refers to the genomic maps and the 10 kb scale to the expanded regions.

Of the 10 complete open reading frames deduced from sequence within the HindIII fragment C of SPV, 5 contain leader sequences consisting of a series of 15-20 hydrophbic residues near the amino terminus which could serve as endoplasmic reticulum transport signals (Wickner and Lodish, 1985). Of these 5 with leaders, 4 have additional hydrophobic stretches characteristic of transmembrane domains which could serve as membrane anchors. In addition, open reading frame C4L and C7L do not have a hydrophobic leader, but rather internal stretches of hydrophobic residues, which could also serve as transport and anchor signals. Therefore, of the 10 complete SPV open reading frames, 7 potentially encode membrane proteins.

Predictions regarding the time of expression of the SPV HindIII C terminal open reading frames can be based on certain features found to be conserved within vaccinia early or late transcription elements. For example, the presence of the early transcript termination motif, TTTTTNT, can be used to predict early expression. Likewise, the presence of the late transcript promotor motif, TAAAT, as decribed by Davison and Moss (1989b), or TAAAAT, as described by Weinrich and Hruby (1986), within 50 bp upstream of the initiating codon can be used to predict that an orf is expressed at late times. Using these criteria, only 4 SPV orf's, C5L, C7L, C8L and C10L have the early termination motif and would be expressed at early times. The regions

upstream of each of these orf's also conform well to the early promotor consensus sequence, AAAAATGAAAA/TA, with the C5L promotor being the most divergent with 4 base changes, C7L showing 2 base changes, and both C8L and C10L showing only a single base difference from the consensus. Alternatively, 8 of the 10 complete orf's have the late transcription motifs, TAAAT or TAAAAT, with only orf's C3L and C6L lacking this consensus sequence. Therefore, one would predict that these 8 open reading frames are all expressed at late times, and that orf's C5L, C7L, C8L and C10L, which exhibit both early and late consensus motifs, are expressed at both early and late times during an infection.

CHAPTER 7

SUMMARY AND CONCLUSIONS

Swinepox virus is reportedly worldwide in distribution and endemic in many swine populations in both developed and underdeveloped countries (Kasza et al., 1960). Previous studies involving SPV have focused primarily on the ultrastructure of the virus and the pathology noted in natural and experimental infections of swine (Shope, 1940; Cheville, 1966; Teppema and De Boer, 1975; Conroy and Meyer, 1971; Meyer and Conroy, 1972). In this study, I have examined various facets of swinepox virus and its gene expression, confirmed that SPV is a unique member of the poxvirus family, and begun a molecular analysis of the virus.

The hybridization of SPV DNA to the DNA of vaccinia and various other poxviruses (Figure 3-1, Panel B) at low stringency failed to reveal any significant gross homology to members of the Orthopoxvirus, Leporipoxvirus, Avipoxvirus, genera or the Entomopoxvirus subfamily. I performed more detailed experiments designed to compare swinepox virus to other poxviruses, particularly vaccinia virus from which much of our current knowledge of these viruses has been obtained. One of these experiments

involved the use of anti-swinepox virus and anti-vaccinia sera to compare the antigenic relationship between these two viruses (Figure 3-2). Immunoprecipitations with the homologous and heterologous antisera revealed little or no antigenic crossreactivity between SPV and vaccinia. These observations are consistent with the lack of cross neutralizing antibody previously reported (Shope, 1940; Schwarte and Biester, 1941; Datt, 1964; De Boer, 1975). These data substantiate the previous classification of swinepox virus into a separate genus, Suipoxvirus, which was originally based primarily on serological evidence alone.

We have determined the restriction maps of SPV for the enzymes HindIII, AvaI, HaeII, KpnI, BglI, SalI and XhoI. The left-to-right orientation of the maps (Figure 5-3) is based on the preliminary mapping of the SPV genes encoding a thymidine kinase and the second largest subunit of the viral RNA polymerase (data not shown), and places these SPV genes in positions analogous to those of like genes in vaccinia. Our reasoning is also based on evidence of a conserved central core of essential genes within poxvirus genomes which is maintained among various genera, and possibly throughout the entire family (Drillien et al., 1981; Binns et al., 1988; Gershon and Black, 1989). The orientation and colinearity of the maps was subsequently confirmed by the DNA sequence analysis of the central region of the SPV genome.

I present data to suggest that the SPV genome contains covalently closed termini (Fig. 5-2) and is approximately 175 kb in length. I am confident that this size estimate, based on the results of pulsed field electrophoresis analysis, is more accurate than the size estimates based on the summation of restriction fragments (shown in Table 5-1). The genomic size as estimated by pulsed field analysis is the same, whether the DNA is purified prior to analysis, or examined as a component of a total cell lysate, digested and analyzed directly from agarose plugs. The size estimate is also the same regardless of the amount of DNA loaded per lane. The disparity in genomic size between the summation of restriction fragment sizes and electrophoretic analysis is most likely due to a variety of reasons including errors inherent in summations of a large number of restriction fragments of varying size, as well as differences in G+C content between SPV DNA and the lambda DNA size standards. The summation of vaccinia, Shope fibroma or myxoma virus restriction fragments determined in parallel with the SPV digests results in similarly low molecular size estimates of 160 kb for vaccinia and 140 kb for Shope fibroma and myxoma viruses. Errors of this nature are minimized in data obtained from pulsed field analysis since this size is deduced from a single molecule and from simultaneous comparisons to other poxvirus DNA which, as a family, all exhibit high A+T content within their genomes.

Recent advances in pulsed field electrophoresis technology have also allowed the resolution and reproducible separation of viral DNA of large sizes not previously possible (Bostock, 1988). I have taken advantage of this resolving power to easily separate and isolate monomers from dimeric replicative DNA to simplify mapping of the terminal fragments (Figure 5-4). The analysis of the SPV genome suggests that the termini contain inverted repetitions of approximately 5 kilobases (Figre 5-6). The data also indicate that many of the characteristic features noted from studies of the genomes of other poxvirus have been conserved within the SPV genome. Direct repeats have been demonstrated within the ITRs of members of the orthopoxvirus (Wittek and Moss, 1980; Pickup et al., 1982) and avipoxvirus (Campbell et al., 1989) groups, but appear to be lacking among the leporipoxviruses (Upton et al., 1987a). By end-labeling the fragments of the HindIII digest of a serially passed population of SPV, I have shown that the termini are heterogenous, consisting of a family of fragments differing from each other by 70 bp (Figure 5-8). Therefore, SPV DNA also appears to contain a varying number of direct repeats. The number of bands suggests that up to 20 repeats may be present, at intervals of approximately 70 bp. This data, as well as the fact that this heterogeneity begins to rapidly reappear upon passage of a plaque purified virus isolate (data not shown), indicates that SPV DNA is highly unstable

and readily undergoes recombination, at least within the ITRs, as has previously been shown for vaccinia and fowlpox virus (Wittek et al., 1978; Tomley et al., 1988).

During the initial work with SPV involving growth of this virus in tissue culture for the isolation of DNA, it soon became obvious that this virus behaved quite differently from the other poxviruses we had encountered. Most notably, cytopathic effect was not evident until approximately 4 days postinfection, in stark contrast to the orthopoxviruses, such as vaccinia virus, for which CPE is readily observable prior to 24 hours (Figure 4-1). The fact that SPV forms foci is not unique as numerous poxviruses exhibit this trait, including vaccinia when grown on a cell line that overexpresses the epidermal growth factor receptor (Buller et al., 1988). SPV will form plaques on swine testes (ST) cells, but once again CPE is not observed until 4 days postinfection.

Additional analyses were subsequently undertaken to further examine the unusual growth characteristics of swinepox virus, as initially suggested by the observed delay in the onset of CPE in tissue culture. The gross expression of viral proteins is difficult to detect prior to 24 hr postinfection whether measuring total protein synthesis by pulse labeling of infected cells (Figure 4-3), or by Coomassie stained total protein patterns (data not shown). By 32 hr viral proteins are clearly detected and their

synthesis continues to increase through 48 hr. However, immunoprecipitation experiments (Figure 4-4) show that late viral proteins are detectable as early as 12 hr postinfection, and early protein synthesis is evident by 4 hr. It appears that the pattern of viral protein synthesis prior to 32 hr is obscured when looking at the total pattern of protein synthesis due to the inability of SPV to effectively shut off host synthesis prior to 24-32 hr postinfection. The delayed shut off of host protein synthesis is independent of both the cytopathology induced and the cell line since the delay is noted on both PK-15, cells where the virus forms foci, and in swine testes cells, where the virus forms plaques. These observations differ markedly from what is seen with vaccinia, in terms of the time post-infection at which late protein synthesis is first observed (4-6 hr postinfection) (Moss and Salzman, 1968; Pennington, 1974), as well as the rapid inhibition of host protein synthesis caused by the virus (Moss, 1968). The slow inhibition of host protein synthesis by SPV may be related to a failure to induce a more rapid turnover of host cell mRNA coupled with a less rapid accumulation of viral mRNA than that observed for vaccinia (Rice and Roberts, 1983).

The infectious cycle of SPV was also analyzed by means of an experiment designed to detect the accumulation of replicated viral DNA. Analogous experiments performed for both SPV and vaccinia reveal that although the initiation of

SPV DNA replication appears to be delayed in comparison to vaccinia, it is in terms of the accumulation of DNA over a 48 hr period that the difference between these two viruses is most striking (Figure 4-2). The accumulation of replicated vaccinia DNA is first detectable at 6 hr postinfection, peaks at 24 hr, and decreases by 48 hr, possibly due to degradation of unpackaged DNA upon deterioration of the host cells. However, for SPV, DNA does not accumulate to detectable levels until about 12 hr postinfection and does not peak until at least 48 hr. The ultimate peak of SPV DNA accumulation may be even later than 48 hr because infections for the purpose of viral DNA isolation are routinely harvested at 4 days postinfection, and at this time there are large quantities of SPV DNA present with no indication of degradation. Recent advances in pulsed field electrophoresis technology has not only made possible my DNA accumulation experiments, but has allowed the development of methodology for the routine isolation of SPV, fowlpox and entomopox virus DNAs, and should be applicable to all poxviruses. When compared to standard purification protocols, this method results in comparable yields but, more importantly, in DNA of superior quality in terms of both integrity and purity.

Another facet of the swinepox virus development I have examined is that of RNA transcription. In general, poxvirus gene expression and RNA synthesis is quite complex and our

knowledge on the subject has been derived mostly from studies of vaccinia virus. RNA expression for simplicity is divided into two phases, with early transcripts being synthesized prior to replication and late mRNAs representing those transcripts synthesized after replication has begun. Early messages are discrete in length and have a structure characteristic of normal eukaryotic mRNA whereas late messages are atypical in the sense that they are extremely heterogeneous in length (Cooper and Moss, 1979). The transcription analysis of SPV reveals that the late messages are also heterogeneous in length and this heterogeneity is first detected at 8 hr postinfection, and becomes quite pronounced by 10 hr (Figure 4-5).

For vaccinia, DNA synthesis initiates at approximately 2 hr postinfection and late mRNA is detectable at 3-4 hr. In our hands, the accumulation of vaccinia DNA was readily observed at 6 hr postinfection. In each case, there is a lag between the initiation of replication and the observed accumulation of DNA, presumably due to differing sensitivities of the two techniques. For SPV, late mRNA was detected at 8 hr and DNA accumulation could be seen at 12 hr. From these data I can estimate that SPV DNA replication is most likely initiated between 5 and 7 hr postinfection. I have attempted radiolabeled thymidine incorporation experiments to more accurately estimate this time but these experiments were unsuccessful due to a high background of

continuing host DNA synthesis, presumably resulting from the fact that SPV infections seem to actually have a transient stimulatory effect on host macromolecular synthesis. It is interesting to speculate that the cause of this stimulation, as well as the foci formation caused by this virus, may be related to the presence of a growth factor as has been clearly demonstrated in vaccinia (Stroobant et al., 1985; Blomquist et al., 1984; Brown et al., 1985; Reisner, 1985; Twardzik et al., 1985; King et al., 1986), and putatively identified by DNA sequence homology in myxoma (Upton et al., 1987b), Shope fibroma virus (Chang et al., 1983;) and fowlpox virus (Campbell et al., 1989). However, any such SPV growth factor will apparently be a member of a different class of growth factors, or be quite divergent from that of vaccinia and the leporipoxviruses, since degenerate oligonucleotides derived from the conserved regions of these epidermal growth factor like genes failed to detect a similar homolog in swinepox virus (R. F. Massung and R.W. Moyer, unpublished observations). In addition, all of the poxviral growth factor homologs identified to date are located near the left terminus of their respective genomes, either within the ITR or just adjacent to it. However, my sequence analysis of this region of SPV DNA has found no open reading frames with homology to any type of known growth factor. In fact, the lack of a functional growth factor may be at least partially responsible for the delayed

kinetics and/or the limited pathogenicity of SPV. Such a theory could be easily tested by inserting the vaccinia growth factor gene into SPV and observing phenotypic changes in tissue culture or in experimental infections of swine.

From these data I have accumulated on the delayed expression of the SPV viral components, I conclude that swinepox virus is unusual among poxviruses that have been well characterized and infect mammalian species. The kinetics of the avipoxviruses are also reportedly "slow", with DNA replication detectable at 12-16 hr postinfection (Prideaux and Boyle, 1987), but productive infections are limited to avian species (Taylor et al., 1988). Tanapox virus is a human pathogen endemic to equatorial Africa (Manson-Bahr and Downie, 1973) that is also reportedly very slow. For example, CPE is not evident in tissue culture cells infected with tanapox virus until 3 to 4 days postinfection (Knight et al., 1989). However, the expression of tanapox genes, and the tanapox replicative cycle, have not been examined in detail and therefore can not be directly compared to what I observe for SPV. An examination of the phylogenetic relationship between SPV and other poxviruses is currently underway and involves a detailed comparative analysis of the thymidine kinase genes of SPV and numerous other poxviruses at the DNA sequence and amino acid level (Feller et al., manuscript in preparation).

The DNA sequence analysis of 2.85 kbp of the central core region of SPV (Figure 6-2) revealed a striking degree of conservation as compared to the analogous region of vaccinia virus. The conservation of three open reading frames included the left-to right orientation, their lengths, and location within their respective genomes (Figure 6-3). The identity at the amino acid level ranged from 69.7% for the partial sequence of the SPV NTPase to 77% for the small subunit of the mRNA capping enzyme. This high degree of homology was rather surprising considering the lack of crosshybridizations of SPV and vaccinia DNAs, as shown in Figure 3-1. However, this apparent discrepancy is due to the high A+T content of the SPV genome, resulting in a T_m (melting temperature) for the 2.85 kb region of approximately 48° C. Since the crosshybridizations were done at 50° C no hybridization would be expected.

Also apparently conserved within SPV are many of the early and late transcription signals. Recent independent data has shown that the swinepox virus thymidine kinase gene and its promotor are functional in a vaccinia virus background (Feller et al., manuscript in preparation) and supports the conservation of transcriptional elements between SPV and vaccinia. The high degree of homology noted between the 2.85 kb region of SPV and the HindIII D fragment of vaccinia, two rather distantly related viruses, provides further evidence that all vertebrate poxviruses contain a

conserved central core element that includes all the genes responsible for maintenance of the unique poxvirus cytoplasmic lifecycle.

The ability of vaccinia virus to grow in the presence of the antibiotic rifampicin was previously mapped to a single nucleotide substitution, resulting in the conversion of an asparagine to an aspartic acid residue (Tartaglia and Paoletti, 1985). The swinepox analogue to the vaccinia rifampicin resistance gene maintains the asparagine residue at amino acid position 480, similar to wild type vaccinia. Although SPV has not been tested for the effect of rifampicin, the prediction would be that it will be sensitive, as is wild type vaccinia.

The analysis of the DNA sequence of the SPV genome near the left terminus identified 10 complete and 1 partial open reading frame (Figure 6-4). These open reading frames are arranged in tandem fashion with the transcription of each directed towards the left terminus, similar to the tandem open reading frames previously described near the termini of other poxvirus family members (Earl and Moss, 1989; Goebel et al., 1990; Upton et al., 1987a). Between the SPV open reading frames are very short (less than 70 bp) intergenic regions, again similar to the open reading frame organization of other poxviruses. However, the SPV open reading frames showed very little homology to those from the analogous region of vaccinia virus DNA, and appear to be

much more closely related to the open reading frames within the ITRs of the leporipoxviruses such as Shope fibroma virus (see Table 6-1). SPV orfs C4L, C5L, C8L, and C11L do show homology to individual vaccinia open reading frames but in vaccinia the analogous orfs are located much more internally, approximately 25-35 kb from the left terminus (Figure 6-6). However, SPV orfs C2L, C4L, and C6L demonstrate significant homology to open reading frames within the ITRs of Shope fibroma virus and the other leporipoxviruses, myxoma and malignant rabbit fibroma virus. SPV orf C4L has approximately 35% amino acid identity with three tandem orfs within the leporipoxvirus ITRs designated C6L, C8L, and C9L. The leporipox ITRs are about 12.5 kb, and the junction between the ITR and unique internal sequence is within orf C9L. For SPV, which has ITRs of less than 5 kb, this junction would fall within orf C4L, the SPV analogue of leporipox orf C9L. One could speculate that SPV and the leporipoxviruses diverged from a common ancestor relatively recently and that the leporipox tandem orfs are the result of duplication of a single orf in the progenitor which was more like SPV. Alternatively, SPV may have diverged from a lepori-like ancestor and derived its single orf (C4L) from a recombinational event between lepori orfs C6L and C9L. However, the former scenario seems more likely since the recombination event would result in the deletion of the intervening sequence, including leporipox open reading frame

C7L, for which SPV retains an analogue (C6L). In the latter scenario, this SPV analogue would have been deleted. It should be noted that vaccinia also has a single copy orf with homology to SPV orf C4L and the leporipox tandem orfs, but again, it is located quite distant compared to the others, approximately 25 kb from the left terminus (Figure 6-6). But the fact that this orf is present in these three divergent genera indicates a common ancestor for all and suggests an advantageous, if not essential, function for the product of this gene.

Despite the similarity between several open reading frames within the ITRs of SPV and the leporipoxviruses, SPV also appears to contain direct repeats near its termini (Figure 5-8), which the leporipox lack (Upton et al., 1987a). However, the DNA near the hairpin termini of vaccinia and cowpox virus does contain several sets of direct repeats, including multiple 70 bp repeat elements (Pickup et al., 1982: Wittek and Moss, 1980), the same size estimated for the repeat elements of SPV. The combined data would indicate that SPV may actually be intermediate between the orthopox and leporipox genera, retaining some features of each; namely, the direct repeats of the orthopoxviruses, and the open reading frames of the leporipoxviruses. However, the actual origin of vaccinia virus is unknown and, although it is capable of experimentally infecting a wide range of animals, there is no known natural host (Fenner et

al., 1989). Vaccinia has also been maintained in tissue culture for numerous decades and it is quite possible that vaccinia is a very poor representative of the orthopoxvirus family, particularly regarding the genetic content near the termini of the genome. Numerous studies have demonstrated that the terminal regions of vaccinia and other orthopoxviruses are dispensible in tissue culture, but important for host range and pathogenic properties in the natural host (Archard et al., 1984; Dumbell and Archard, 1980; Fenner, 1958; McClain, M.E., 1965; Moyer et al., 1980; Perkus et al., 1990a; Perkus et al., 1990b; Pickup et al., 1984). Therefore, one must be very cautious when making comparisons of the terminal regions of vaccinia to natural isolates of other viruses. A much more relevent comparison would be with natural isolates of other orthopoxviruses such as ectromelia or smallpox.

When comparing the open reading frames deduced from the terminal region of SPV to those of vaccinia and the leporipoxviruses, the most striking difference is in the predicted temporal transcription pattern. Each of the Shope fibroma virus open reading frames located within its 12.5 kb ITR contain the consensus early termination motif, TTTTTNT, and are therefore predicted to be expressed at early times during infection. Similarly, nearly all of the vaccinia open rading frames within its ITR are also expressed early. This constrasts radically with our predictions concerning the

expression of the terminal SPV genes. Only 4 of the 10 complete open reading frames contain the early termination motif, while 8 of contain the consensus late promotor motifs within 50 bp of the predicted translation initiation codon.

Interestingly, 8 of the 10 complete SPV open reading frames within the terminal region sequence also potentially code for membrane bound or secreted glycoproteins (Table 6-2). Two of these (C3L and C6L) have significant homology to cellular receptors while one orf (C1L) has an RGD cell adhesion tripeptide. One could speculate that C1L may be involved in viral attachment to host cells. This hypothesis could be tested by determining whether synthetic RGD tripeptides, or longer polypeptides that also contain the amino acids flanking the RGD sequence of C1L, are capable of neutralizing the infectivity of the virus. Alternatively, mutagenesis of the RGD tripeptide would also be useful for testing the cell adhesion properties of the RGD motif in swinepox virus. The homology of SPV polypeptides C3L and C6L to cellular receptors suggests that they may play a role in pathogenesis by involvement in a signal transduction pathway, thereby manipulating host cell regulatory mechanisms. It is interesting to speculate that one, or both, of these polypeptides, due to their potential involvement in host regulatory mechanisms, may be the SPV functional analogue of the growth factors found in other poxviruses but apparently missing from swinepox virus. If

this hypothesis is true, the deletion of the sequences encoding these open reading frames from the virus should result in drastic changes in the cytopathology resulting from infections in tissue culture cells and in the natural host. Specifically, one would pedict that the focus-forming ability of the virus will be altered. Subsequent mutagenesis studies of these open reading frames would subsequently be useful for elucidating the functional domains of the polypeptides, expanding our present knowledge regarding structure-function relationships of signal transducton proteins, in general, and more specifically, those involved in the modifications of host regulatory mechanisms resulting from poxvirus infections.

The significance of these unique findings regarding the terminal open reading frames of SPV are numerous. Characteristically, early poxvirus genes are expressed at low levels and the encoded proteins are often difficult to detect, whereas late genes and their products are expressed at high levels. From our data, the same appears to be true for swinepox virus (see Figures 4-3 and 4-4). Presumably this is the case because many of the late poxvirus genes encode structural components required in significant quantities for the synthesis and morphogenesis of mature virions. In vaccinia and other examined poxviruses these genes are located in the conserved central core region of the genome, while the termini encode non-structural genes

expressed at early times. However, SPV appears to differ in this regard encoding primarily membrane bound polypeptides expressed at late times near its termini. Being late, the products of these genes are likely to be expressed in high quantities. For the genes within the inverted terminal repeats, C1L through C4L, the expression should even be higher due to the duplicate copies present at each end of the genome. And if our predictions concerning the membrane localization of most of the mature polypeptide products are correct, the implications regarding the potential makeup of SPV virions and alterations of the host cell are fascinating. For example, one could envision the SPV virion being composed of not only the components common to all poxviruses, but including numerous members of these terminally encoded products. This could explain the previously noted lack of neutralization between SPV and other poxvirus family members. The involvement of these products in cell attachment could explain the host range specificity of SPV, and may be responsible for limiting its tissue tropism by sterically blocking or replacing normal poxvirus membrane components with others of more stringent tissue specificity. Alternatively, the products of these terminal open reading frames could have little to do with the composition of intracellular naked virions (INV's), but could be inserted into the membrane of the host cell. This would drastically alter the protein composition of the host

membrane, and likely alter cellular function as well. Induced alterations of either the cell adhesion or signal transduction properties of the host cell, properties for which SPV contains homologues of normal cellular factors, could explain the foci formation induced by SPV. With this scenario, the products expressed on the host membrane could subsequently be incorporated into the virion by the formation of extracellular enveloped virions (EEV's) during budding of the virus, although the presence or absence of EEV's for SPV has not been explored. Likewise, future studies will be required to fully examine the potential of the SPV terminal gene products and to determine their contributions to viral pathogenicity and host range.

It must be noted that these predicted open reading frames are based soley on DNA sequence data. Confirmation of the expression and function of these open reading frames must await future biochemical and genetic analyses. However, from experience with other poxviruses, most, if not all, potential poxvirus open reading frames are expressed (Moss, 1990). Several small (less than 100 amino acid) potential orfs were not presented in the data because they were each embedded in other large orfs, usually transcribed in the opposite direction, and did not agree with the preferred SPV codon usage.

In conclusion, the evidence presented here indicates that SPV is a unique and extremely interesting member of the

poxvirus family. The classification of SPV into a separate genus is appropriate based on DNA hybridization, immunological, and DNA sequence data. The kinetics of the virus are unusually slow for unknown reasons. The genome has been characterized paving the way for future analyses of SPV and its utilization as a vehicle for furthering our overall understanding of the family Poxviridae, as well as its development and utilization as a live vaccine vector for swine.

REFERENCES

Aguet, M., Dembic, Z., and Merlin, G. (1988). Molecular cloning and expression of the human interferon gamma receptor. Cell 55:273-280.

Archard, L.C., Mackett, M., Barnes, D.E., and Dumbell, K.R. (1984). The genome structure of cowpox virus white pock mutants. J. Gen. Virol. 65:875-886.

Aviv, H., and Leder, P. (1972). Purification of biologically active globin messenger RNA by chromatography on oligothymidylic acid-cellulose. Proc. Natl. Acad. Sci. USA 69:1408-1412.

Baldick, C.J.Jr., and Moss, B. (1987). Resistance of vaccinia virus to rifampicin coferred by a single nucleotide substitution near the predicted NH_2 terminus of a gene encoding a M_r 62,000 polypeptide. Virology 156:138-145.

Bankier, A.T., and Barrell, B.G. (1983). In "Techniques in the Life Sciences," Biochemistry-Volume B5:B508. Elsevier Scientific Publishers Irelzhangand Ltd., County Clare, Ireland.

Baroudy, B.M., Venkatesan, S., and Moss, B. (1982). Incompletely base-paired flip-flop terminal loops link the two DNA strands of the vaccinia virus genome into one uninterrupted polynucleotide chain. Cell 28:315-324.

Bauer, W.R., Ressner, E.C., Kates, J., and Patzke, J.V. (1977). A DNA nicking-closing enzyme encapsidated in vaccinia virus: partial purification and properties. Proc. Natl. Acad. Sci. USA 74:1841-1845.

Bearcroft, W.G.C., and Jamieson, M.F. (1958). Yaba tumor virus. An outbreak of subcutaneous tumours in rhesus monkeys. Nature 182:195-196.

Bertholet, C., Van Meir, E., ten Heggeler-Bordier, B., and Wittek, R. (1987). Vaccinia virus produces late mRNAs by discontinuous synthesis. Cell 50:153-162.

Binns, M.M., Boursnell, M.E.G., Tomley, F.M., and Campbell, J. (1989). Analysis of the fowlpoxvirus gene encoding the 4b core polypeptide and demonstration that it possesses efficient promotor sequences. Virology 170:288-291.

Binns, M.M., Tomley, F.M., Campbell, J., and Boursnell, M.E. (1988). Comparison of a conserved region in fowlpox virus and vaccinia virus genomes and the translocation of the fowlpox thymidine kinase. J. Gen. Virol. 69:1275-1283.

Blomquist, M.C., Hunt, L.T., and Barker, W.C. (1984). Vaccinia virus 19-kilodalton protein: Relationship to several mammalian proteins, including two growth factors. Proc. Natl. Acad. Sci. USA 81:7363-7367.

Bonner, W.M., and Laskey, R.A. (1974). A film detection method for tritium-labelled proteins and nucleic acids in polyacrylamide gels. Eur. J. Biochem. 46:83-88.

Bostock, C.J. (1988). Parameters of field inversion gel electrophoresis for the analysis of pox virus genomes. Nucleic Acids Res. 16:4239-4252.

Boursnell, M.E.G., Foulds, I.J., Campbell, J.I., and Binns, M.M. (1988). Non-essential genes in the vaccinia virus HindIII K fragment: A gene related to serine protease inhibitors and a gen related to the 37K vaccinia virus major envelope antigen. J. Gen. Virol. 69:2995-3003.

Boyle, D.B., and Coupar, B.E.H. (1986). Identification and cloningof the fowlpox thymidine kinase gene using vaccinia virus. J. Gen. Virol. 67:1591-1600.

Boyle, D.B., and Coupar, B.E.H. (1988). Construction of recombinant fowlpox viruses as vectors for poultry vaccines. Virus Res. 10:343-356.

Brown, J.P., Twardzik, D.R., Marquardt, H., and Todaro, G.J. (1985). Vaccinia virus encodes a polypeptide homologous to epidermal growth factor and transforming growth factor. Nature 313:491-492.

Brown, S.T., Nalley, J.F., and Kraus, S.J. (1981). Molluscum Contagiosum. Sex. Transm. Dis. 8:227-234.

Broyles, S.S., and Moss, B. (1987). Identification of the vaccinia virus gene encoding nucleoside triphosphatase phosphohydrolase I, a DNA-dependent ATPase. J. Virol. 61:1738-1742.

Buller, R.M., Chakrabarti, S., Moss, B., and Fredrickson, T. (1988). Cell proliferative response to vaccinia virus is mediated by VGF. Virology 164:182-192.

Campbell, J.I., Binns, M.M., Tomley, F.M., and Boursnell, M.E. (1989). Tandem repeated sequences within the terminal region of the fowlpox virus genome. J. Gen. Virol. 70:145-154.

Carrasco, L. and Bravo, R. (1986). Specific proteins synthesized during the viral lytic cycle in vaccinia virus infected HeLa cells: Analysis by high-resolution two-dimensional gel electrophoresis. J. Virol. 58:569-577.

Challberg, M. D., and Englund, P.T. (1979). Purification and properties of the deoxyribonucleic acid polymerase induced by vaccinia virus. J. Biol. Chem. 254:7812-7819.

Chang, A., and Metz, D.H. (1976). Further investigations on the mode of entry of vaccinia virus into cells. J. Gen. Virol. 32:275-282.

Chang, W., Upton, C., Hu, S., Purchio, A.F., and McFadden, G. (1986). The genome of Shope fibroma virus, a tumorigenic poxvirus, contains a growth factor gene with sequence similarity to those encoding epidermal growth factor and transforming growth factor alpha. Mol. Cell. Biol. 7:535-540.

Cheville, N.F. (1966). The cytopathology of swine pox in the skin of swine. Am. J. Pathol 49:339-352.

Chirgwin, J.M., Przybyla, A.E., MacDonald, R.J., and Rutter, W.J. (1979). Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry 18:5294-5299.

Conroy, J.D., and Meyer, R.C. (1971). Electron microscopy of swinepox virus in germfree pigs and in cell culture. Am. J. Vet. Res. 32:2021-2032.

Cooper, J.A., and Moss, B. (1979). In vitro translation of immediate early, early, and late classes of RNA from vaccinia virus-infected cells. Virology 96:368-380.

Cooper, J.A., Wittek, R., and Moss, B. (1981). Extension of the transcriptional and translational map of the left end of the vaccinia virus genome to 21 kilobase pairs. J.Virol. 39:733-745.

Coupar, B.E.H., Boyle, D.B., and Both, G.W. (1987). Effect of in vitro mutations in a vaccinia virus early promotor region monitored by herpes simplex virus thymidine kinase expression in recombinant vaccinia virus. J. Gen. Virol. 68:2299-2309.

Dales, S. (1973). Early events in cell-animal virus interactions. Bacteriol. Rev. 37:103-135.

Dales, S. and Kajioka, R. (1964). The cycle of multiplication of vaccinia virus in Earle's strain L cells. I. Uptake and penetration. Virology 24:278-294.

Dales, S., and Pogo, B.G. (1981). Biology of poxviruses. Virol. Monogr. 18:1-109.

Datt, N.S. (1964). Comparative studies of pig pox and vaccinia viruses. I. Host range pathogenicity. J.Comp.Pathol. 74:62-68.

Davison, A.J., and Moss, B. (1989a). Structure of vaccinia virus early promotors. J. Mol. Biol. 210:749-769.

Davison, A.J., and Moss, B. (1989b). Structure of vaccinia virus late promotors. J. Mol. Biol. 210:771-784.

De Boer, G.F. (1975). Swinepox. Virus isolation, experimental infections and the differentiation from vaccinia virus infections. Arch.Virol. 49:141-150.

Dedhar, S., Ruoslahti, E., and Pierschbacher, M.D. (1987). A cell surface receptor complex for collagen type 1 recognises the Arg-Gly-Asp sequence. J. Cell Biol. 104:585-593.

DeLange, A.M. (1989). Identification of temperature-sensitive mutants if vaccinia virus that are defective in conversion of concatemeric replicative intermediates to the mature linear DNA genome. J.Virol. 63:2437-2444.

DeLange, A.M., Macaulay, C., Block, W., Mueller, T., and McFadden, G. (1984). Tumorigenic poxviruses: construction of the composite physical map of the Shope fibroma virus genome. J.Virol. 50:408-416.

Devereux, J., Haeberli, P., and Smithies, O. (1984). A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. 12:387-395.

Drillien, R., Koehren, F., and Kirn, A. (1981). Host range deletion mutant of vaccinia virus defective in human cells. Virology 111:488-499.

Drillien, R., Spehner, D., Villeval, D., and Lecocq, J.P. (1987). Similar genetic organization between a region of fowlpox virus DNA and the vaccinia virus HindIII J fragment despite divergent location of the thymidine kinase gene. Virology 160:203-209.

Dumbell, K.R., and Archard, L.C. (1980). Comparison of white pock (h) mutants of monkeypox virus with parental monkeypox and with variola-like viruses isolated from animals. Nature 286:29-32.

Earl, P.L., Jones, E.V., and Moss, B. (1986). Homology between DNA polymerase of poxviruses, herpesviruses, and adenoviruses: nucleotide sequence of the vaccinia virus DNA polymerase gene. Proc. Natl. Acad. Sci. 61:3659-3663.

Earl, P.L., and Moss, B. (1989). Vaccinia virus. Genetic maps 1989. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Ensinger, M.J., Martin, S.A., Paoletti, E., and Moss, B. (1975). Modification of the 5'-terminus of mRNA by soluble guanylyl and methyl transferases from vaccinia virus. Proc. Natl. Acad. Sci. USA 72:2525-2529.

Esposito, J., Condit, R., and Obijeski, J. (1981). The preparation of orthopoxvirus DNA. J. Virol. Methods. 2:175-179.

Esposito, J.J., and Knight, J.C. (1985). Orthopoxvirus DNA: A comparison of restriction profiles and maps. Virology 143:230-251.

Essani, K., and Dales, S. (1979). Biogenesis of vaccinia: Evidence for more than 100 polypeptides in the virion. Virology 95:385-394.

Feinberg, A.P., and Vogelstein, B. (1983). A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. 132:6-13.

Fenner, F. (1958). The biological characteristics of several strains of vaccinia, cowpox and rabbitpox virus. Virology 5:502-529.

Fenner, F., Wittek, R., and Dumbell, K.R. (1989). "The Orthopoxviruses". Academic Press, New York.

Fox, G., Parry, N.R., Varnett, P.V., McGinn, B., Rowlands, D.J., and Brown, F. (1989). The cell attachment site on foot-and-mouth disease virus includes the amino acid sequence RGD (arginine-glycine-aspartic acid). J. Gen. Virol. 70:625-637.

Fuchs, R. (1990) Free molecular biological software available from the EMBL fileserver. Comput. Appl. Biosci. 6:120-121.

Garg, S.K., and Meyer, R.C. (1972). Adaptation of swinepox virus toan established cell line. Appl.Microbiol. 23: 180-182.

Gartner, T.K. and Bennett, J.S. (1985). The tetrapeptide analogue of the cell attachment site of fibronectin inhibits platelet aggregationand fibrinogen binding to activated platelets. J. Biol. Chem. 260:11891-11894.

Gershon, P.D., and Black, D.N. (1989). A capripox pseudogene whose only intact homologs are in other poxvirus genomes. Virology 172:350-354.

Gillard, S., Spehner, D., Drillien, R., and Kirn, A. (1986). Localization and sequence of a vaccinia virus gene required for multiplication in human cells. Proc. Natl. Acad. Sci. U.S.A. 83:5573-5577.

Gilman, A.G. (1987). G proteins: transducers of receptor-generated signals. Annu. Rev. Biochem. 56:615-649.

Glisin, V., Crkvenjakov, R., and Byus, C. (1974). Ribonucleic acid isolated by cesium chloride centrifugation. Biochemistry 13:2633-2637.

Goebel, S.J., Johnson, G.P., Perkus, M.E., Davis, S.W., Winslow, J.P., and Paoletti, E. (1990). The complete nucleotide sequence of vaccinia virus. Virology 179:247-266.

Haverstick, D.M., Cowan, J.F., Yamada, K.M., and Santoro, S.A. (1985). Inhibition of platelet adhesion to fibronectin, fibrinogen, and von Willebrand factor substrates by a synthetic tetrapeptide derived from the cell-binding domain of fibronectin. Blood 66:946-952.

Hayman, E.G., Pierschbacher, M.D., and Ruoslahti, E. (1985).Detachment of cells from culture substrate by soluble fibronectin peptides. J. Cell Biol. 100:1948-1954.

Hirt, P., Hiller, G., and Wittek, R. (1986). Localization and fine structure of a vaccinia virus gene encoding an envelope antigen. J. Virol. 58:757-764.

Johnson, D.A., Gautsch, J.W., Sportsman, J.R., and Elder, J.H. (1984). Improved technique utilizing nonfat dry milk for analysis of proteins and nucleic acids transferred to nitrocellulose. Gene Anal. Tech. 1:3-3.

Jones, E.V., and Moss, B. (1984). Mapping of the vaccinia virus DNA polymerase gene by marker rescue and cell-free translation of selected RNA. J. Virol. 49:72-77.

Kasza, L., Bohl, E.H., and Jones, D.O. (1960). Isolation and cultivation of swinepox virus in primary cell cultures of swine origin. Am.J.Vet.Res. 21:269-273.

Kasza, L., and Griesemer, R.A. (1962). Experimental swine pox. Am.J.Vet.Res. 23, 443-450.

Kates, J.R., and McAuslan, B.R. (1967). Poxvirus DNA-dependent RNA polymerase. Proc. Natl. Acad. Sci. USA 58:134-141.

Keck, J.G., Baldick, C.J.Jr., and Moss, B. (1990). Role of DNA replication in vaccinia virus gene expression: A naked template is required for transcription of three late trans-activator genes. Cell 61:801-809.

King, C.S., Cooper, J.A., Moss, B., and Twardzik, D.R. (1986). Vaccinia virus growth factor stimulates tyrosine protein kinase activity of A431 cell epidermal growth factor receptors. Mol. Cell Biol. 6:332-336.

Klein, P., Kanehisa, M., and DeLisi, C. (1985). The detection and classification of membrane spanning proteins. Biochim. Biophys. Acta 815:468-476.

Knight, J.C., Novembre, F.J., Brown, D.R., Goldsmith, C.S., and Esposito, J.J. (1989). Studies on Tanapox virus. Virology 172:116-124.

Kotwal, G.J., and Moss, B. (1988a). Vaccinia virus encodes a secretory polypeptide structurally related to complement control proteins. Nature 335:176-178.

Kotwal, G.J., and Moss, B. (1988b). Analysis of a a large cluster of nonessential genes deleted from a vaccinia virus terminal transposition mutant. Virology 167:524-537.

Kotwal, G.J., and Moss, B. (1989). Vaccinia virus encodes two proteins that are structurally related to members of the plasma serine protease inhibitor superfamily. J. Virol. 63:600-606.

Kushner, S.R. (1978). An improved method for transformation of Escherichia coli with ColEI derived plasmids. In:"Genetic Engineering" (H.W. Boyer and S. Nicosia, Eds.) pp. 17-23. Elsevier, North Holland, Amsterdam.

Lehrach, H., Diamond, D., Wozney, J.M., and Boedtker, H. (1977). RNA molecular weight determinations by gel electrophoresis under denaturing conditions, a critical reexamination. Biochemistry 16:4743-4751.

Mackett, M., and Archard, L.C. (1979). Conservation and variation in orthopoxvirus genome structure. J. Gen. Virol. 45:683-701.

Mackett, M., and Smith, G.L. (1986). Vaccinia viris expression vectors. J. Gen. Virol. 67:2067-2082.

Mackett, M., Smith, G.L., and Moss, B. (1982). Vaccinia virus: a selectable eukaryotic cloning and expression vector. Proc. Natl. Acad. Sci. USA 70:7415-7419.

Maniatis, T., Fritsch, E.F., and Sambrook, J. (1982). Molecular cloning: A laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Manson-Bahr, P.E.C., and Downie, A.W. (1973). Persistence of Tanapox in Tana River Valley. Br. Med. J. 2:151-153.

Mars, M., and Beaud, G. (1987). Characterization of vaccinia virus early promotors and evaluation of their information content. J. Mol. Biol. 198:619-631.

Martin, S.A., and Moss, B. (1975). Modification of RNA by mRNA guanylyltransferase and mRNA (guanine-7-) methyltransferase from vaccinia virions. J. Biol. Chem. 250:9330-9335.

Martin, S.A., and Moss, B. (1976). mRNA guanylyltransferase and mRNA (guanine-7-)methyltransferase from vaccinia virions. Donor and acceptor substrate specificities. J. Biol. Chem. 251:7313-7321.

Martin, S.A., Paoletti, E., and Moss, B. (1975). Purification of mRNA guanylyltransferase and mRNA (guanine-7-) methyltransferase from vaccinia virions. J. Biol. Chem. 250:9322-9329.

Matthews, R.E.F. (1982). Classification and nomenclature of viruses. Intervirology 17:42-46.

McClain, M.E. (1965). The host range and plaque morphology of rabbitpox virus (RPu+) and its u mutants on chick fibroblast, PK-2a, and L929 cells. Aust. J. Exp. Biol. Med. Sci. 43:31-44.

McGeoch, D.J. (1990). Protein sequence comparisons show that the pseudoproteases encoded by poxviruses and certain retroviruses belong to the deoxyuridine triphosphatase family. Nucleic Acids Res. 18:4105-4110.

Mercer, A.A., Fraser, K.M., Stockwell, P.A., and Robinson, A.J. (1989). A homologue of retroviral peseudoproteases in the parapoxvirus, orf virus. Virology 172:665-668.

Messing, J., Gronenborn, B., Muller-Hill, B., and Hofscheider, P.H. (1977). Filamentous coliphage M13 as a cloning vehicle: Insertion of a HindII fragment of the lac regulatory region in M13 replicative form in vitro. Proc. Natl. Acad. Sci. USA 74:3642-3646.

Messing, J., and Vieira, J. (1982). A new pair of M13 vectors for selecting either DNA strand of double-digest restriction fragments. Gene 19:269-276.

Meyer, R.C., and Conroy, J.D. (1972). Experimental swinepox in gnotobiotic piglets. Res. Vet. Sci. 13:334-338.

Moss, B. (1968a). Inhibition of HeLa cell protein synthesis by the vaccinia virion. J. Virol. 2:1028-1037.

Moss, B. (1990). Poxviridae and Their Replication. In:" Virology" (Fields, B.N., Ed.) pp. 2079-2112. Raven Press, New York.

Moss, B., Ensinger, M.J., Martin, S.A., and Wei, C.M. (1975b). Modification of the 5'-terminus of mRNA by guanylyl and methyl transferases from vaccinia virus. In: Haenni, A.L., Beaud, G., ed. In vitro transcription and translation of viral genomes. Paris, INSERM. 161-168.

Moss, B., and Flexner, C. (1987). Vaccinia virus expression vectors. Annu. Rev. Immunol. 5:305-324.

Moss, B., Gershowitz, A., Wei, C.M., and Boone, R. (1976). Formation of the guanylylated and methylated 5'-terminus of vaccinia virus mRNA. Virology 72:341-351.

Moss, B., Rosenblum, E.N., and Paoletti, E. (1973). Polyadenylate polymerase from vaccinia virions. Nature New. Biol. 245:59-63.

Moss, B., and Rosenblum, E.N. (1974). Vaccinia virus polyriboadenylate polymerase: convalent linkage of the product with polyribonucleotide and polydeoxyribonucleotide primers. J. Virol. 14:86-98.

Moss, B., Rosenblum, E.N., and Gershowitz, A. (1975a). Characterization of a polyriboadenylate polymerase from vaccinia virions. J. Biol. Chem. 250:4722-4729.

Moss, B., and Salzman, N.P. (1968b). Sequential protein synthesis following vaccinia virus infection. J. Virol. 2:1016-1027.

Moyer, R.W., and Graves, R.L. (1981). The mechanism of cytoplasmic orthopoxvirus DNA replication. Cell 27:391-401.

Moyer, R.W., and Rothe, C.T. (1980). The white pock mutants of rabbit poxvirus. I. Spontaneous host range mutants contain deletions. Virology 102:119-132.

Munyon, W.H., and Kit, S. (1966). Induction of cytoplasmic ribonucleic acid synthesis in vaccinia-infected LM cells during inhibition of protein synthesis. Virology 29:303-306.

Munyon, W., Paoletti, E., and Grace, J.T.Jr. (1967). RNA polymerase activity in purified infectious vaccinia virus. Proc. Natl. Acad. Sci. USA 58:2280-2287.

Niles, E.G., Condit, R.C., Caro, P., Davidson, K., Matusick, L., and Seto, J. (1986). Nucleotide sequence and genetic map of the 16kb vaccinia virus HindIII D fragment. Virology 153:96-112.

Niles, E.G., Lee-Chen, G.J., Shuman, S., Moss, B., and Broyles, S.S. (1989). Vaccinia virus gene D12L encodes the small subunit of the viral mRNA capping enzyme. Virology 172:513-522.

Oie, M., and Ichihashi, Y. (1981). Characterization of vaccinia polypeptides. Virology 113:263-276.

Paoletti, E. (1977). In vitro synthesis of a high molecular weight virion-associated RNA by vaccinia. J. Biol. Chem. 252:866-871.

Panicali, D., and Paoletti, E. (1982). Construction of poxviruses as cloning vectors: Insertion of the thymidine kinase gene from herpes simplex virus into the DNA of infectious vaccinia virus. Proc. Natl. Acad. Sci. USA 79,4927-4931.

Pearson, W.R., and Lipman, D.J. (1988). Improved tools for biological sequence comparison. Proc. Natl. Acad. Sci. 85:2444-2448.

Pennington, T.H. (1974). Vaccinia virus polypeptide synthesis: sequential appearance and stability of pre- and post-replicative polypeptides. J. Gen. Virol. 25:433-444.

Perkus, M.E., Goebel, S.J., Davis, S.W., Johnson, G.P., Limbach, K., Norton, E.K., and Paoletti, E. (1990a). Vaccinia virus host range genes. Virology 179:276-286.

Perkus, M.E., Goebel, S.J., Davis, S.W., Johnson, G.P., Norton, E.K., and Paoletti, E. (1990b). Deletion of 55 open reading frames from the termini of vaccinia virus. Virology 180:406-410.

Pickup, D.J., Bastia, D., Stone, H.O., and Joklik, W.K. (1982). Sequence of terminal regions of cowpox virus DNA: Arrangement of repeated and unique sequence elements. Proc. Natl. Acad. Sci. USA 79:7112-7116.

Plow, E.F., Pierschbacher, M.D., Ruoslahti, E., Marguerie, G.A., and Ginsberg, M.H. (1985). The effect of Arg-Gly-Asp-containing peptides on fibrinogen and von Willebrand factor binding to platelets. Proc. Natl. Acad. Sci. USA 82:8057-8061.

Pogo, B.G., Freimuth, P., and Stein, A. (1982). Shope fibroma virus. I. Biological and molecular properties of a cytocidal and a noncytocidal strain. J. Virol. 41:97-103.

Prideaux, C.T., and Boyle, D.B. (1987). Fowlpox virus polypeptides: sequential appearance and virion associated polypeptides. Arch. Virol. 96:185-199.

Reisner, A.H. (1985). Similarity between the vaccinia virus 19K early protein and epidermal growth factor. Nature 313:801-803.

Relman, D., Tuomanen, E., Falkow, S., Golenbock, D.T., Saukkonen, K., and Wright, S.D. (1990). Recognition of a bacterial adhesin by an integrin: Macrophage CR3 ($\alpha_M\beta_2$, CD11b/CD18) binds filamentous hemagglutinin of Bordetella pertussis. Cell 61:1375-1382.

Rice, A.P., and Roberts, B.E. (1983). Vaccinia virus induces cellular mRNA degradation. J. Virol. 47:529-539.

Rodriguez, J.F., Kahn, J.S., and Esteban, M. (1986). Molecular cloning, encoding sequence, and expression of vaccinia virus nucleic acid-dependent nucleoside triphosphatase gene. Proc. Natl. Acad. Sci. USA 83:9566-9570.

Roizman, B., and Batterson, W. (1985). Herpesviruses and their replication. In:"Virology" (Fields, B.N., Ed.) pp. 607-636. Raven Press, New York.

Rosel, J.L., Earl, P.L., Weir, J.P., and Moss, B. (1986). Conserved TAAATG sequence at the transcriptional and translational sites of vaccinia virus late genes deduced by structural and functional analysis of the HindIII genomic fragment. J. Virol. 60:436-449.

Roseman, N.A., and Slabaugh, M.B. (1990). The vaccinia virus HindIII F fragment: Nucleotide sequence of the left 6.2 kb. Virology 178:410-418.

Ruoslahti, E., and Pierschbacher, M.D. (1986). Arg-Gly-Asp: A versatile cell recognition signal. Cell 44:517-518.

Russell, R.J., and Robbins, S.J. (1989). Cloning and molecular characterization of the myxoma virus genome. Virology. 170:147-159.

Sanger, F., Coulson, A.R., Barrell, B.G., Smith, A.J.H., and Roe, B.A. (1980). Cloning in single-stranded bacteriophage as an aid to rapid DNA sequencing. J. Mol. Biol. 143:161-178.

Sanger, F., Nicklen, S., and Coulson, A.R. (1977). DNA sequencing with chain terminating inhibitors. Proc. Natl. Acad. Sci. U.S.A. 74:5463-5468.

Schnitzlein, W.M., Ghildyal, N., and Tripathy, D.N. (1988). Genomic and antigenic characterization of avipoxviruses. Virus. Res. 10:65-75.

Schwarte, L.H., and Biester, H.E. (1941). Pox in swine. Am. J. Vet. Res. 2:136-140.

Schwer, B., Visca, P., Vos, J.C., and Stunnenberg, H.G. (1987). Discontinuous transcription or RNA processing of vaccinia virus late messengers results in a 5' poly(A) leader. Cell 50:163-169.

Seto, J., Celenza, L.M., Condit, R.C., and Niles, E.G. (1987). Genetic map of the vaccinia HindIII D fragment. Virology 160:110-119.

Shope, R.E. (1932). A transmissible tumor-like condition in rabbits. J. Exp. Med. 56:793-802.

Shope, R.E. (1940). Studies from the Rockefeller Institute for Medical Research. Arch. Gesamte. Virusforsch. 1:457-467.

Shuman, S. and Moss, B. (1987). Identification of a vaccinia virus gene encoding a type I DNA topoisomerase. Proc. Natl. Acad. Sci. USA 84:7478-7482.

Shuman, S., Surks, M., Furneaux, H., and Hurwitz, J. (1980). Purification and characterization of a GTP-pyrophosphate exchange activity from vaccinia virions. Association of the GTP-pyrophosphate exchange activity with vaccinia mRNA guanylyltransferase. RNA (guanine-7-)methyltransferase complex (capping enzyme). J.Biol.Chem. 255:11588-11598.

Slabaugh, M.B., and Roseman, N.A. (1989). Retroviral protease-like gene in the vaccinia virus genome. Proc. Natl. Acad. Sci. USA 86:4152-4155.

Slabaugh, M.B., Roseman, N.A., and Mathews, C.K. (1988). Vaccinia-virus encoded ribonucleotide reductase: sequnce conservation of the gene for the small subunit and its amplification in hydroxyurea-resistant mutants. J. Virol. 62:519-527.

Southern, E.M. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98:503-517.

Spiess, M., and Lodish, H.F. (1986). An internal signal sequence: the asialoglycoprotein receptor membrane anchor. Cell 44:177-185.

Springer, W.R., Cooper, D.N.W., and Barondes, S.H. (1984). Discoidin I is implicated in cell-substratum attachment and ordered cell migration of Dictyostelium discoidium and resembles fibronectin. Cell 39:557-564.

Staden, R. (1986). The current status and portability of our sequence handling software. Nucleic Acids Res. 14:217-231.

Stroobant P., Rice, A.P., Gullick, W.J., Cheng, D.J., Kerr, I.M., and Waterfield, M.D. (1985). Purification and characterization of vaccinia virus growth factor. Cell 42:383-393.

Stryer, L., and Bourne, H.R. (1986). G proteins: A family of signal transducers. Annu. Rev. Cell Biol. 2:391-419.

Tabor, S., and Richardson, C.C. (1987). DNA sequence analysis with a modified bacteriophage T7 DNA polymerase. Proc. Natl. Acad. Sci. USA 84:4767-4771.

Tamkun,J.W., DeSimone, D.W., Fonda, D., Patel, R.S., Buck, C., Horwitz, A.F., and Hynes, R.O. (1986). Structure of integrin, a glycoprotein involved in the transmembrane linkage between fibronectin and actin. Cell 46:271-282.

Tartaglia, J., and Paoletti, E. (1985). Physical mapping and DNA sequence analysis of the rifampicin resistance locus in vaccinia virus. Virology 147:394-404.

Tartaglia, J., Pincus, S., and Paoletti, E. (1990). Poxvirus-based vectors as vaccine candidates. Crit. Rev. Immunol. 10:13-30.

Taylor, J., and Paoletti, E. (1988). Fowlpox virus as a vector in non-avian species. Vaccine 6:466-468.

Taylor, J., Weinberg, R., Kawaoka, Y., Webster, R.G., and Paoletti, E. (1988). Protective immunity against avian influenza induced by a fowlpox virus recombinant. Vaccine 6:504-508.

Tengelsen, L.A., Slabaugh, M.B., Bibler, J.K., and Hruby, D.E. (1988). Nucleotide sequence and molecular genetic analysis of the large subunit of ribonucleotide reductaseencoded by vaccinia virus. Virology 164:121-131.

Teppema , J.S., and De Boer, G.F. (1975). Ultrastructural aspects of experimental swinepox with special reference to inclusion bodies. Arch. Virol. 49:151-163.

Tomley, F., Binns, M., Campbell, J., and Boursnell, M. (1988). Sequence analysis of an 11.2 kilobase, near terminal, Bam HI fragment of fowlpox virus. J. Gen. Virol. 69:1025-1040.

Traktman, P., Sridhar, P., Condit, R.C., and Roberts, B.E. (1984). Transcriptional mapping of the DNA polymerase gene of vaccinia virus. J. Virol. 49:125-131.

Twardzik, D.R., Brown, J.P., Ranchalis, J.E., Todaro, G.J., and Moss, B. (1985). Vaccinia virus-infected cells release a novel polypeptide functionally related to transforming and epidermal growth factors. Proc. Natl. Acad. Sci. USA 82:5300-5304.

United States Biochemical Corporation. Step-by-step Protocols for DNA sequencing with Sequenase™. P.O. Box 22400, Cleveland, Ohio.

Upton, C., DeLange, A.M., and McFadden, G. (1987a). Tumorigenic poxviruses: Genomic organization and DNA sequence of the telomeric region of the Shope fibroma virus genome. Virology 160:20-30.

Upton, C., Macen, J.L., and McFadden, G. (1987b). Mapping and sequencing of a gene from myxoma virus that is related to those encoding epidermal growth factor and transforming growth factor alpha. J. Virol. 61:1271-1275.

Upton, C., Macen, J.L., Wishart, D.S., and McFadden, G. (1990). Myxoma virus and malignant rabbit fibroma virus encode a serpin-like protein important for virus virulence. Virology 179:618-631.

Upton, C., and McFadden, G. (1986). DNA sequence homology between the inverted terminal repeats of Shope fibroma virus and an endogenous cellular plasmid species. Mol. Cell. Biol. 6:265-276.

Vos, J.C., and Stunnenberg, H.G. (1988). Depression of a novel class of vaccinia virus genes upon DNA replication. EMBO J. 7:3487-3492.

Wei, C.M., and Moss, B. (1974). Methylation of newly synthesized viral messenger RNA by an enzyme in vaccinia virus. Proc. Natl. Acad. Sci. USA 71:3014-3018.

Wei, C.M., and Moss, B. (1975). Methylated nucleotides block 5'-terminus of vaccinia virus messenger RNA. Proc. Natl. Acad. Sci. USA 72:318-322.

Weinrich, S.L., and Hruby, D.E. (1986). A tandemly-oriented late gene cluster within the vaccinia virus genome. Nucleic Acids Res. 14:3003-3016.

Weir, J.P., and Moss, B. (1984). Regulation of expression and nucleotide sequence of a late vaccinia virus gene. J. Virol. 51:662-669.

Weir, J.P., and Moss, B. (1987). Determination of the promotor region of an early vaccinia virus gene encoding thymidine kinase. Virology 158:206-210.

Wickner, W.T., and Lodish, H.F. (1985). Multiple mechanisms of protein insertion into and across membranes. Science 230:400-407.

Wills, A., DeLange, A.M., Gregson, C., Macaulay, C., and McFadden, G. (1983). Physical characterization and molecular cloning of the Shope fibroma virus DNA genome. Virology. 130:403-414.

Wittek, R., Hanggi, M., and Hiller, G. (1984a). Mapping of a gene coding for a major late structural polypeptide on the vaccinia virus genome. J. Virol. 49:371-378.

Wittek, R., and Moss, B. (1980). Tandem repeats within the inverted terminal repetition of vaccinia virus DNA. Cell 21:277-284.

Wittek, R., Muller, H.K., and Wyler, R. (1978). Length heterogeneity in the DNA of vaccinia virus is eliminated on cloning the virus. FEBS. Lett. 90:41-46.

Wittek, R., Richner, B., and Hiller, G. (1984b). Mapping of the genes coding for the two major vaccinia virus core polypeptides. Nucleic Acids Res. 12:4835-4848.

Woodson, B. (1967). Vaccinia mRNA synthesis under conditions which prevent uncoating. Biochem. Biophys. Res. Commun. 27:169-175.

World Health Organization (1980). Hist. Int. Public Health, No.4. W.H.O., Geneva.

Wright, S.D., Reddy, P.A., Jong, M.T.C., and Erickson, B.W. (1987). C3bi receptor (complement receptor type 3) recognizes a region of complement protein C3 containing the sequence Arg-Gly-Asp. Proc. Natl. Acad. Sci. USA 84:1965-1968.

Yuen, L., and Moss, B. (1987). Oligonucleotide sequence signaling transcriptional termination of vaccinia virus early genes. Proc. Natl. Acad. Sci. USA 84:6417-6421.

Zhang, H., Scholl, R., Browse, J., and Somerville, C. (1988). Double stranded DNA sequencing as a choice for DNA sequencing. Nucleic Acids Res. 16:1220.

BIOGRAPHICAL SKETCH

The author was born in McKeesport, Pennsylvania, on May 22, 1953. He graduated from McKeesport High School in 1971 and attended Pennsylvania State University, majoring in marketing, for three years but withdrew after his junior year. He subsequently found employment with the U.S. Steel Corporation and worked in numerous positions ranging from laborer to millwright until 1982. It was during this time that he married his high school sweetheart Marcia Stegman, and they had their first two children, Robert Patrick and Breanne Lynn. In 1982 Robert decided to continue his aborted education and entered the field of science. In 1986 he graduated from California University of Pennsylvania with a bachelor's degree in medical technology. In 1985 Marcia and Robert's third child, Valerie Anne, was born. Having whetted his appetite for science education, Robert and family moved to Gainesville in 1986, and he enrolled in the graduate program of the Department of Immunology and Medical Microbiology. There he joined the lab of Dick Moyer and worked on several interesting projects culminating in the analysis of swinepox virus presented here. Robert and family will be moving to Atlanta, Georgia, where he will continue his study of poxviruses, having been awarded a National

Research Council associateship to work with Dr. Joseph Esposito at the Centers for Disease Control.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Richard W. Moyer, Chair
Professor of Immunology and Medical Microbiology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Paul A. Gulig
Assistant Professor of Immunology and Medical Microbiology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Henry V. Baker
Assistant Professor of Immunology and Medical Microbiology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Carl M. Feldherr
Professor of Anatomy and Cell Biology

This dissertation was submitted to the Graduate Faculty of the College of Medicine and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

May, 1991

Roger H. Nein

Dean, College of Medicine

Madelyn Lockhart

Dean, Graduate School